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EXPERIMENTS CONCERNING THE POSSIBILITY THAT INULIN IS SECRETED BY THE RENAL TUBULES[1]

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Impressive evidence exists in support of belief that the plasma clearance of the polysaccharide, inulin, is a reliable measure of the rate of glomerular filtration in mammals. It has been found to be completely filtrable from plasma through artificial membranes impermeable to proteins and through the frog's glomerular membrane (1). It is not excreted by the aglomerular kidneys of certain teleost fishes (2). Its plasma clearance in all species of animals studied is independent of its concentration in the plasma (3), in this respect differing from phenol red (4) or the organic iodine compounds, diodrast and hippuran (5) which are in part secreted by the tubules. In normal dogs and rabbits the clearances of inulin and creatinine are alike (6); ferrocyanide clearance is equal to these in normal dogs (7). In phlorhizinized animals the clearances of inulin, creatinine, glucose, xylose and sucrose are equal (8). Since it is scarcely credible that tubular processes of secretion and reabsorption could deal identically with such a variety of substances, these observations and coincidences are regarded as proof that glomerular filtration is the sole process concerned in the renal excretion of inulin in mammals.

Clearance measurements in normal and phlorhizinized man have agreed so well with those in laboratory animals as to justify belief that the human kidney excretes inulin solely by filtration (9). Calculations of rates of glomerular filtration from inulin clearances in normal adult man (1.73 sq.m. body surface area) yielded values of about 120 cc. per minute (10). In dogs, the value per square meter is about the same, 60 to 70 cc. per minute. These volumes of plasma filtrate appear to be sufficient to contain

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all of the solutes contained in normal urine which are not formed within the kidney.

The fundamental importance of the magnitude of these values and the growing volume of work which involves reliance on their validity made it seem not superfluous to seek additional experimental evidence concerning the possibility that inulin is in part secreted by the mammalian tubule, for it is in respect of this aspect of the question that mistaken belief would lead to most serious confusion.

The experiments described in this paper represent such attempts carried out on frogs, dogs and rabbits. They give no indication that the tubules of these animals can secrete inulin.

Frog experiments. Inulin, intravenously injected into frogs, is excreted in the glomerular filtrate in the same concentration as in the plasma and in higher concentration in the urine (1). A similar statement is true of the perfused kidney when inulin is present in the arterial perfusion fluid.

To learn whether inulin can be excreted by the tubules, the kidneys were perfused by the double perfusion method of Cullis as developed by later experimenters, inulin being added to the renal portal perfusion fluid only. Numerous experiments by Höber and his colleagues (11) have shown the method to be competent to reveal secretion of certain dyes and of uric acid (12) by the frog's tubules.

METHODS. Two species of frogs were used: *R. catesbiana* and *R. pipiens*. The brain and spinal cord were destroyed by pithing. The circulation was maintained during the necessary dissections either by repeated injections of small amounts of Ringer's solution into the anterior abdominal vein or by connecting the aortic perfusion cannula, as soon as possible after the body cavity was opened, with the aortic perfusion bottle. (The aortic cannula, pointing toward the heart, was inserted just posterior to the renal arteries.) The renal portal veins were perfused via the anterior abdominal vein. Urine was collected through ureteral cannulas attached to horizontally-placed 1 cc. pipettes.

The perfusion fluids ("Ringer's solution") were made with glass-distilled water and the purest obtainable reagents according to the formula of Barkan, Broemser and Hahn (13) and were saturated with a mixture of 98.5 per cent O_2 + 1.5 per cent CO_2. The pH of different lots ranged from 7.5 to 7.8. The chloride concentration of this fluid, expressed as NaCl, was 0.685 per cent.

The majority of experiments were made with a preparation of inulin obtained from the Bureau of Standards.

The perfusion pressures varied in different experiments. In the majority the aortic pressure was between 26 and 42 cm. of water. Renal portal pressure was never more than one-half the height of the aortic; usually about one-third.

Inulin was determined by the Shaffer-Somogyi method (14) applied before and after hydrolysis with N/10 H_2SO_4. When the concentrations were low, the accuracy of the procedure was increased by the addition of known amounts of levulose to the fluids. Chloride was determined by the Whitehorn method (15); uric acid and creatinine by the methods of Folin (16).

RESULTS. In 17 tests the aorta was perfused with Ringer's solution; the renal portal veins with Ringer containing 150–500 mgm. per cent

of inulin. In 6, the inulin content of the urine was zero; in 7, the concentration was less than 5 mgm. per cent; in 2 it was between 5 and 10 mgm. per cent: and in 2 it was 24 and 25 mgm. per cent. The chloride concentration of these urines averaged less than two-thirds of that of the perfusion fluids; the glucose concentration, less than one-fifth.

In one of the experiments uric acid (as sodium urate) and creatinine were added to the renal portal perfusion fluid to make the concentration of each 5 mgm. per cent; the inulin concentration was 150 mgm. per cent. The urine contained no inulin; 37 mgm. per cent of uric acid; 2.5 mgm. per cent of creatinine. This result accords with Lueken's conclusion (12) that the frog's tubule has the capacity of secreting uric acid. In another experiment phenol red (5 mgm. per cent) was added to the inulin-containing renal portal perfusion fluid. The urine contained no inulin, 11.9 mgm. per cent of phenol red. These experiments show that frog's tubules which were capable of reabsorbing chloride and glucose and of secreting uric acid or phenol red did not secrete inulin.

The results of the 2 experiments mentioned, in which the urinary inulin values were 24 and 25 mgm. per cent, and the small positive values in others raised some doubt as to the finality of the answer which the experiments as a group seemed to provide. It seemed highly improbable that the appearance of such small amounts of inulin in the urine could represent the remnants of a secretory process in the tubules; for in one of the two exceptional experiments the urine, which contained 24 mgm. per cent of inulin, was excreted late in an experiment, in an earlier similar period of which no inulin was excreted. Damage resulting from prolonged perfusion was probably responsible. It was further found in 8 experiments that the addition of NaCN M/1500 – M/500 to the inulin-containing renal portal perfusion fluid always resulted in the appearance of markedly increased amounts of inulin in the urine. In one experiment, for example, the urine collected after a 20-minute period of renal portal perfusion with M/500 NaCN contained 20 mgm. per cent of inulin; whereas that collected before the CN period contained none.

To make additionally certain that in the CN experiments the inulin entered the tubule through its wall rather than through the glomerulus via the efferent vessel, experiments were made in which horse serum was added to the inulin-containing renal portal perfusion fluid in order to make the colloid osmotic pressure of the fluid greater than the perfusion pressure and so to prevent the formation of a filtrate if the renal portal fluid should reach glomeruli through the efferent vessels. To avoid possible dilution of the colloid which would result if the renal portal and aortic fluids should mix in the glomerular capillaries the aortic perfusion flow in some experiments was made to alternate in three-minute periods with the renal portal perfusion flow. The details of one of these experiments are given below.

Experiment 12. Period I, control, 50 minutes. Aorta perfused with Ringer + 0.05 per cent glucose at 41.5 cm. pressure; renal portal veins perfused at 11.5 cm. pressure with a mixture of equal parts of the aortic fluid and sterile normal horse serum, to which were added inulin to make 500 mgm. per cent and creatinine to make 30 mgm. per cent. The protein concentration was 3.33 per cent. Both perfusions were constant, not alternating.

Period II. Cyanide, 27 minutes. Aortic fluid as in period I. Renal portal fluid is Ringer + NaCN to make M/500. Pressures as in period I.

Period III. Post-cyanide, 73 minutes. Perfusion fluids as in period I. Aortic perfusion at 41.5 cm. pressure alternating every three minutes with renal portal perfusion at 9 cm.

	Period I	*Period III*
Arterial perfusion	95 cc. per hour	63.5 cc. per hour
Renal portal perfusion	54 cc. per hour	32.5 cc. per hour
Urine flow	1.61 cc. per hour	1.26 cc. per hour
NaCl in urine	0.36 per cent	0.62 per cent
Glucose in urine	6.3 mgm. per cent	41.0 mgm. per cent
Inulin in urine	2.4 mgm. per cent	15.0 mgm. per cent
Creatinine in urine	7.3 mgm. per cent	4.1 mgm. per cent

The inulin value of urine I is so near the error of the analytical method as to be insignificant. The inference is justified that only when the frog's tubule is damaged is it permeable to inulin. Creatinine entered the urine through the tubule wall in both periods. The low concentrations indicate that it was not secreted (Höber, 17). The lower concentration in urine III compared with urine I can be explained by its diffusion out of the tubule during the 3-minute periods of aortic perfusion only.

The outcome of this group of experiments yields these conclusions: 1, the undamaged frog's tubule does not permit inulin to pass through its cells into the lumen either by secretion or diffusion; 2, when the tubule is damaged, as a result either of the artificial conditions associated with perfusion or of the action of CN, small amounts of inulin may diffuse from the fluid in the peritubular capillaries into the fluid in the lumen of the tubule.[2]

Mammalian experiments. We have made three groups of experiments on dogs and rabbits in all of which the aim was to discover whether inulin, supplied to the kidney through the renal arteries at a blood pressure too low to permit filtration, can find access to the lumina of the tubules. They are therefore all variants of the experiment which Heidenhain designed to reveal tubular secretion of indigo carmine and uric acid (18). As in the frog experiments, in order to be able to affirm that the experimental conditions had not destroyed the capacity of tubule cells to secrete, the behavior of other substances, now known to be excreted by the tubule

[2] The experiments also lessen the validity of objections to the double perfusion method as a means of identifying tubular secretion in frogs, such as those raised by Richards and Walker, this Journal **116:** 128, 1936.

was studied simultaneously with inulin. These were phenol red and the organic iodine compounds, diodrast and hippuran. Efforts to identify inulin histologically in the lumina of tubules were unsuccessful, even in kidneys excised while excreting it in high concentration and fixed by the method of Holton and Bensley (19). Therefore, at the conclusion of the low pressure inulin perfusion period, the kidneys were perfused, either artificially or naturally, at high pressure with Ringer's solution or with blood containing no added inulin in order to obtain urine specimens which could be expected to contain any substance which had been deposited and retained in the lumina of the tubules or in the tubule cells during the low pressure period. The different procedures employed represent increasingly effective attempts to preserve the integrity of tubule functions to the highest degree compatible with experimental requirements.

Analytical methods. In blood or extracts of kidney tissue inulin was determined as the difference in reducing power of iron filtrates (Steiner, Urban and West, 20), before and after hydrolysis, by the Shaffer-Somogyi method (14); in urine, iron precipitation was usually omitted. Creatinine was determined in the same fluids according to Folin. When phenol red was present, creatinine was first adsorbed on, then released from, Lloyd's reagent according to Gaebler (21). The resulting solution contained some phenol red. An alkaline watery solution of phenol red of the same concentration was prepared by matching in a Duboscq colorimeter. Alkaline picrate was then added to the unknown creatinine solution, to the standard creatinine solutions, to the phenol red solution and to water. Color development was read with the compensating Bürker colorimeter, the compensating cup on the side opposite to the unknown being filled with the phenol red mixture instead of the usual water blank.[3]

Phenol red was determined by the method of Marshall and Vickers (22); urea by the method of Van Slyke and Cullen (23). Skiodan, diodrast and hippuran[4] were determined as iodine by the method of Leipert as described by Elsom, Bott and Shiels (5).

I. In this group are 8 experiments, 6 with dogs, 2 with rabbits. The blood pressure was reduced to 25–30 mm. Hg: in dogs, by destruction of the cord and withdrawal of blood; in rabbits, by partial occlusion of the aorta anterior to the renal arteries. Inulin, phenol red and usually creatinine were then injected, care being taken that the blood pressure should not rise above 30 mm. After a low pressure period of 10 to 40 minutes the vessels of one kidney were then perfused at high pressure until urine samples were obtained in volume believed to be sufficient to wash out the tubules. In 3 experiments (1 dog, 2 rabbits) the perfusion fluid was warm oxygenated Locke's solution injected into the renal artery with a large syringe; in these, urine formed promptly. In the others,

[3] By this method 6 determinations of creatinine in known mixtures with phenol red gave a maximum error of 3 per cent.

[4] Diodrast is 3:5-diiodo-4-pyridon-N-acetic acid diethanolamine (50 per cent iodine); hippuran is sodium ortho-iodohippurate (38.3 per cent iodine).

heparinized diluted dog's blood was used, delivered into the renal artery by a perfusion pump; in these the urine formed slowly.

Finally, inulin, creatinine and phenol red were added to the perfusing blood and enough urine obtained to show whether the kidney had retained its power to concentrate these substances.

One experiment of the series is summarized below. The results are qualitatively similar in all; this one is chosen because in it the tubules exhibited the highest power of excreting phenol red.

Dog 16, 8.3 K. Etherized for 17 minutes, during which tracheotomy was performed, the vagi sectioned, the carotids tied and the brain and cord pithed. Artificial respiration was begun and the anesthetic discontinued. In the following 30 minutes the gastro-intestinal tract was excised after ligation of its arteries and the portal vein; loose ligatures were placed about the left renal artery, left renal vein, right renal artery and vein and the abdominal aorta, posterior to the left kidney; a cannula was inserted into the left ureter. Forty minutes later, blood pressure being 80 mm. Hg, 131 cc. of blood were drawn from the carotid. Blood pressure fell to 30 mm. In the meantime, 170 cc. of blood were taken from the femoral artery of another normal dog under local anesthesia. The two blood collections were kept fluid with heparin.

As soon as the blood pressure had fallen to 30 mm., 20 cc. of a solution containing inulin, 1.0 gram, creatinine, 0.11 gram, and phenol red, 0.04 gram, was infused into the jugular vein. Blood pressure did not exceed 32 mm.

Twelve to fifteen minutes after the end of the injection, the right kidney was tied off for later excision and analysis and a blood sample was taken (blood I); cannulas were inserted into the left renal vein and artery. The artery was connected with a perfusion pump, filled with the animal's own heparinized normal blood, drawn 20 minutes earlier, diluted 2:3 with Locke's solution. Later, the second dog's blood similarly diluted was used. Perfusion continued for 50 minutes at pressures which varied from 88 to 168 mm. Blood collected from the renal vein was re-oxygenated and returned to the pump as needed.

Urine was formed slowly for the first 30 minutes of perfusion, 40 minutes being required to collect the first specimen, 2.3 cc. The second specimen of 2.5 cc. was obtained in 11 minutes.

When the 2 samples of urine had been obtained inulin, creatinine and phenol red were added to the blood in the pump and perfusion continued until 6.5 cc. of urine were obtained, the first 1.25 cc. being discarded. A sample of this blood was taken for analysis (blood II).

The results are shown in table 1.

The figures show that phenol red was avidly taken up by the kidney; inulin was not. A relatively large amount of the deposited phenol red was carried out in the urine; only a relatively minute amount of inulin. Even the small amount of inulin may not all have been derived from that extracted during the low pressure period. The normal blood perfused at high pressure was recirculated; hence there was opportunity for the injected substances, retained in the kidney at the end of the low pressure period, to be washed out by the perfusing blood and excreted in the urine

by filtration. Error from this source is small with respect to phenol red but may be large with respect to inulin and creatinine.

The amounts of phenol red contained in urines I and II were nearly the same. This seems to mean that the dye was contained in cells rather than in lumina of tubules (cf. Marshall and Vickers, 22). In another experiment, however, nearly 95 per cent of the phenol red excreted in the two successive urines was contained in the first sample (cf. Gersh, 24).

TABLE 1

	INULIN			PHENOL RED			CREATININE		
	mgm. per cent		*mgm.*	*mgm. per cent*		*mgm.*	*mgm. per cent*		*mgm.*
(1) Injected dose			1000			40			110
(2) Plasma I	233			2.4			4.3		
(3) Contained in cortex*	64		13.4	18.2		3.8	8.8		1.8
(4) Ratio, Cortex/Plasma I.		0.27			7.6			2.0	
(5) Urine I, 2.3 cc.	122		2.8	27.6		0.64	9.3		0.2
(6) Urine II, 2.5 cc.	61		1.5	24.6		0.62	7.7		0.2
(7) Total, Urines I and II			4.3			1.26			0.4
(8) As per cent of amount injected		0.4			3.15			0.36	
(9) As per cent of amount in cortex		32.1			33.2			22.2	
(10) As clearances of plasma I (cc.)		1.8			52.5			9.3	
(11) Plasma II	80			1.2			5.5		
(12) Urine III, 5.0 cc.	395		19.8	27.1		1.36	63.5		3.18
(13) Concentration ratios, U/P		4.9			22.5			11.5	

* These figures were derived from analyses of the right kidney, the vessels of which were tied off at the end of the low pressure period.

Creatinine was injected simultaneously with the substances in which we were primarily interested with the expectation that it would behave like inulin. The figures in the table indicate that in one respect it did not. Its extraction from the blood in passage through the kidney was in every instance greater than that of inulin, as judged by the ratio of creatinine in the renal cortex to that in blood plasma at the end of the low pressure period. It is more readily diffusible than inulin (25) and we attribute the greater extraction to this fact.

The figures for creatinine in the urine, like those for inulin, are probably misleadingly high, not only for the reason given in connection with inulin, but also because of the residue of endogenous creatinine which we must

assume was present in the kidney at the beginning of the low pressure period. We can not conclude therefore that creatinine was secreted during that period.

The figures for the second part of the experiment in which the three substances tested were present in quantity in the blood with which the kidney was perfused at high pressure show that its power to excrete them in higher concentration than that in the plasma had been retained. The concentration ratio of creatinine is higher than that of inulin (see below).

II. The defects of experiments such as that just described led to others of a different design. In those presented in this section an isolated, low-pressure perfusion of one kidney (the left) was conducted *in situ* in such fashion that normal circulation through the other (the right) was not seriously disturbed. The perfusing blood which contained the substances

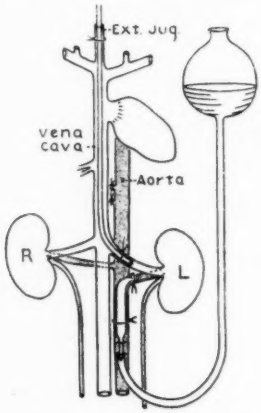

Fig. 1. To illustrate method of perfusing the left kidney in experiments of group II.

to be tested did not mix with the blood in the rest of the animal. The interval between interruption of the normal circulation and the beginning of the perfusion of the left kidney was about 30 seconds; at the end of the low pressure perfusion, the normal circulation through the left kidney was reëstablished without any delay. The arrangements are illustrated in figure 1.

Dogs were anesthetized with nembutal and the gastrointestinal tract excised after ligation of its arteries and the portal vein. Cannulas were inserted into both ureters. The abdominal aorta was clamped below the origin of the left renal artery and a cannula, pointing toward the heart, was tied into it with an elastic ligature. This cannula is bent close to the tip nearly at a right angle and the size of the tip is suited for insertion into the renal artery.

The perfusion fluid was blood, drawn from an artery a few moments before beginning the perfusion, kept fluid with heparin. To it was added about one-tenth of its volume of a solution containing inulin, creatinine and phenol red. A portion was set aside for analysis; the rest used for filling the perfusion bulb, cannula and connecting tube.

The perfusion reservoir was a Dewar bulb of about 100 cc. capacity, with an outlet tube at the bottom. It was connected with the aortic cannula by a jacketed glass tube, through the jacket of which water at 40°C. was circulated. The bulb was supported so that the fluid level was about 37 cm. above the level of the kidney. When the arrangements described in the next paragraph were ready, the perfusion bulb, connecting tube and cannula were filled with the blood-inulin-etc. mixture, a strong clamp placed on the short rubber tubing which connected the cannula with the tube from the bulb, the clamp on the aorta removed and the cannula pushed headward in the aorta until the tip reached the level of the left renal artery. The tip was manipulated into the mouth of the artery and tied into it with one knot in a ligature previously laid about it.

The blood issuing from the left renal vein was collected as follows. A thin-walled metal tube, 55 cm. long, 3 mm. inside diameter, slightly bent near one end, was tied into the right external jugular vein with an elastic ligature. It had previously been filled with oil and one end closed with a piece of rubber tubing and a clamp. The tube was pushed tailward in the vein, so that its tip, passing through the superior into the inferior vena cava, reached the level of the left renal vein. As soon as the tip of the aortic cannula had been tied into the left renal artery, the metal tube was pushed on into the left renal vein and tied in with a ligature previously placed. Then, when the clamp was removed from the perfusion system, the warm blood-inulin-etc. mixture flowed by gravity into the left renal artery and was collected from the left renal vein through the metal tube which projected from the jugular vein in the neck.

When the perfusion was finished, the arterial cannula was withdrawn and instantly the normal arterial blood supply was resumed. Escape from the renal vein was permitted for a few moments in order to wash out the vessels, whereupon the venous cannula was withdrawn, thus reëstablishing the normal venous return from the kidney to the general circulation.

To minimize vasoconstriction in the perfused kidney and thus to secure maximum distribution of the perfusing blood within the kidney, the left splanchnic nerves were usually cut immediately after the evisceration.

As a rule, during the perfusion slightly more blood escaped from the renal vein than entered the renal artery, indicating that a little blood from the high pressure systemic circulation found its way through collaterals into the low pressure renal perfusion.

Blood pressure from a carotid artery was recorded throughout the experiment. Surprisingly little disturbance was caused by the insertion of the metal tube which served as the renal vein cannula.

Urine flowed continuously from the right kidney during the whole period of low pressure perfusion of the left; none from the left. Very soon after reëstablishment of the normal circulation through the perfused kidney, urine began to be excreted by it. Urine samples from both the left and the right kidneys were then collected simultaneously until it was judged that any substance secreted by the left kidney during the low pressure perfusion period should have been eliminated.

Finally, to test the capacities of both kidneys, inulin, creatinine and phenol red were injected intravenously and their simultaneous clearances determined.

The following is a protocol of one experiment.

Experiment 23. December 23, 1935. Dog, 11.4 K. Dissections required 94 minutes. One hundred and forty-one cubic centimeters of blood was taken from a carotid. Blood pressure sank from 148 to 84, then rose to 93 mm. Inulin, creatinine and phenol red were added to the blood; 20 cc. were reserved for analysis (= blood I).

Four and one-half minutes were required for inserting the arterial and venous cannulas, filling and connecting the perfusion system. The circulation in the left kidney was interrupted for half a minute. Perfusion continued for 10½ minutes. One hundred and five cubic centimeters of the blood-inulin mixture flowed into the left renal artery; 107 cc. were recovered from the left renal vein (blood II). After the arterial cannula had been withdrawn, 103 cc. of the animal's blood was allowed to escape through the venous cannula before the cannula was withdrawn (blood III).

In the following 9 minutes, 245 cc. of Ringer's solution were injected intravenously to combat the effect of accidental temporary hemorrhage from the cut abdominal aorta. The blood pressure which had fallen to 79 rose to 122, then fell to 77 mm. Three samples of urine were collected from each kidney after normal circulation in the left had been reëstablished.

I (13 minutes)	left, 1.7 cc.	right, 4.1 cc.
II (9½ ")	left, 2.5 cc.	" 3.26 cc.
III (13½ ")	left, 2.25 cc.	" 4.6 cc.

Plasma in blood I = 69.0 per cent: in II, 68.7 per cent: in blood III, 67.2 per cent.

The calculations from the analytical results are given in table 2.

In this experiment the behavior of inulin, perfused under circumstances which prevented filtration, differed strikingly from that of phenol red with respect to its deposition in the kidney, its release into normal blood subsequently perfused and its excretion into urine subsequently formed. Twenty per cent of the inulin, 50 per cent of the phenol red present in the affluent blood was removed by the kidney; but while 42 per cent of the deposited inulin was quickly washed out by normal blood only 7.6 per cent of the deposited phenol red was taken out. Fixation of inulin by the kidney is not comparable to that of phenol red.

Urine from the kidney which had been perfused with inulin, subsequently formed from blood to which none had been added, contained a minute amount of it (1.2 mgm.) scarcely more, however, than did that formed by the other kidney which had not been perfused (0.8 mgm.). From this it seems certain that inulin, taken up by the blood from the perfused kidney, was distributed to both and excreted by both by glomerular filtration. It is doubtful that any entered the urine through the wall of the tubule.

The behavior of phenol red in this respect is strikingly different. Sixty-three per cent of that which had been deposited in the left kidney was excreted in the urine from it; 8 per cent was excreted by the right kidney in which none had been deposited. These results leave no doubt that the deposited phenol red was excreted through the wall of the tubules.

The figures for creatinine in this experiment indicate that its extraction during the low-pressure perfusion is of the same order as that of inulin and that it is released into the blood with equal readiness. Creatinine excretion in the post-perfusion periods was greater than that of inulin, presumably because of the presence of endogenous creatinine in the blood, in addition to that taken up from the perfused kidney. The fact that the right, unperfused kidney excreted more than the left points to glomerular filtration rather than tubular secretion as the mode of excretion.

In five other similar experiments, similar contrasts between the behavior of inulin and phenol red were observed. In three the deposition of phenol red in the kidney was greater, being from 79 to 88 per cent of that which passed in with the perfusing blood. The amounts excreted by the perfused kidney as percentages of amounts extracted by it were, for phenol red, from 16 to 40 per cent; for inulin 0.7 to 6.4 per cent.

TABLE 2

	INULIN		CREATININE		PHENOL RED	
	mgm.		*mgm.*		*mgm.*	
In with blood I	205.6		23.5		4.71	
Out with blood II	164.6		18.4		2.35	
Extracted by kidney	41.0 (19.9%)		5.1 (21.7%)		2.36 (50.1%)	
Washed out in blood III	17.3		2.1		0.18	
Left in kidney	23.7 (11.5%)		3.0 (12.7%)		2.18 (46.3%)	
	Left	Right	Left	Right	Left	Right
Excreted in urine I	0.95	0.82	0.79	1.12	1.14	0.06
Excreted in urine II	0.2	0	0.53	0.70	0.20	0.065
Excreted in urine III	0.05	0	0.43	1.06	0.04	0.051

The equality of the percentages of extracted creatinine and inulin in the above experiment is exceptional. In five other experiments, the extracted percentage of creatinine was from one-fourth to four-fifths greater than the corresponding percentage of retained inulin. In the three of these in which the wash-out blood was analyzed the amounts of creatinine finally left in the kidney, similarly expressed, were 78, 188 and 573 per cent greater than those of inulin. From this we conclude that, while inulin and creatinine behave quite differently from phenol red, when all are supplied to the kidney in blood at low pressure, inulin penetrates the tissue less readily and is given up more readily than is creatinine.

As a final stage in the experiment, a mixture of inulin, creatinine and phenol red was injected intravenously and urine collected for 11 minutes. Blood was taken 4 minutes after the injection and again at the end of the urine period, mixed in equal parts and the plasma analyzed. Rate

of urine flow from the left kidney was 0.1 cc. per minute; from the right 0.345 cc. per minute. Plasma clearances were low compared with those of a normal dog. Calculated from inulin, clearance by the left kidney was 4.5 cc. per minute; by the right, 15.1 cc. per minute. Concentrating power was well retained; the concentration ratios for the left and right kidneys were: inulin, 45 and 44; creatinine, 57 and 66; phenol red, 45 and 43. The concentration ratios of creatinine are higher than those of inulin. This was also true of the corresponding periods in 3 out of 4 of the other experiments of this group and of those in 4 experiments of group I. The obvious implication, in view of existing evidence of the identity of inulin and creatinine clearances in normal dogs, is that some creatinine was secreted. Being aware that the experiments were not specifically designed for most accurate comparisons of clearances, it seems unwise to adopt this as the explanation or to attempt to suggest an alternative.

III. A third group of 6 experiments was made with rabbits, anesthetized with urethane, in which, after evisceration, blood pressure in one kidney (the left) was reduced to below the filtration level by an adjustable clamp[5] applied to the abdominal aorta between the origins of the right and left renal arteries. It was tightened so that the pressure in the arteries peripheral to it, including the left renal, was lowered to about 25 mm. Hg as measured by a manometer connected with one of the iliac arteries. The substances whose excretion was to be measured, dissolved in from 6 to 15 cc. of saline, were then injected through a jugular vein. During the ensuing period of from 10 to 15 minutes the low blood pressure in the left kidney was maintained. Urine was collected from the right kidney, in one experiment as a single specimen for the whole period; in the others, as several successive specimens. Blood for analysis was taken during this period; in two experiments as a composite sample, 1 cc. per minute; in 4 experiments as single specimens twice during the period. No urine was excreted by the left kidney during this time.

The clamp on the aorta was then released. As soon as urine was seen to be flowing in the left ureteral cannula (one-half to two minutes after release of the clamp) simultaneous urine collections from both kidneys were begun and continued for from 6 to 13 minutes. A sample of blood was taken during or at the end of this period. In two experiments 10 cc. of Ringer's solution were injected after the clamp had been released in order to increase rate of urine flow.

It was found in other experiments that the aortic clamp, adjusted as described, reduced the blood flow through the left kidney to about one-fourth of its previous rate. (To minimize this reduction, the left splanchnic nerves were cut immediately after the evisceration.) It is assumed

[5] A very satisfactory adjustable clamp was constructed from a manipulator described by one of us (J. Biol. Chem. **87**: 463, 1930).

that the low pressure in the left kidney abolished glomerular filtration; that the decreased blood flow reduced, but did not abolish, tubular secretion; there was no interference with either process in the right kidney. It is also assumed that when the clamp was released, filtration promptly recommenced in the left kidney and that the urine then elaborated from this filtrate would carry out the substances secreted during the low pressure period, in addition to those secreted during the after periods. Comparison

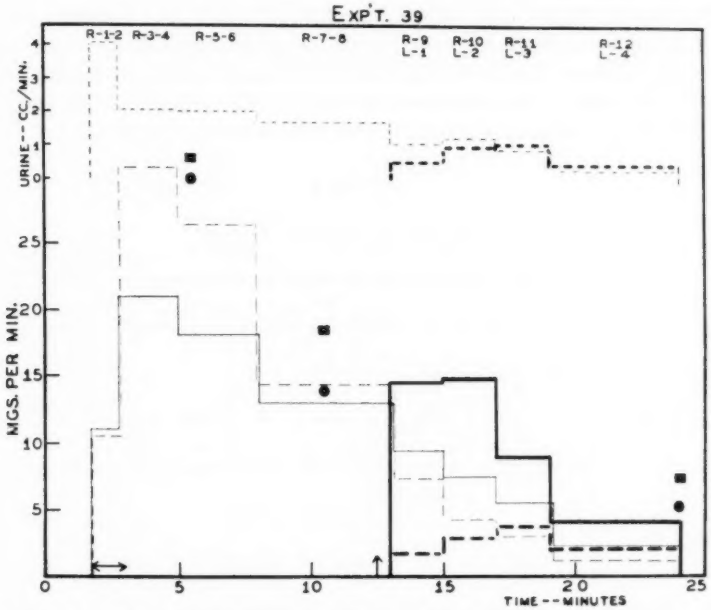

Fig. 2. Records of experiment 39. The broken lines represent rates of urine (upper) and inulin (lower) excretion; light, by the right kidney; heavy, by the left kidney. Unbroken lines represent rates of phenol red excretion; light, by the right; heavy, by the left kidney. Solid circles show plasma phenol red concentrations, mgm. per 100 cc. × 5. Solid squares show plasma inulin concentrations, mgm. per 100 cc. × 0.1.

of rates of excretion by the two kidneys during the after periods should therefore inform us not only whether any of the injected substances had been deposited in the left kidney in such a situation as to be washed out by subsequently formed urine (secreted) but should also permit a rough calculation of the amount of secretion if any occurred.

Figure 2 is a chart of one complete experiment showing rates of excretion by the two kidneys and plasma concentrations of the injected inulin and

phenol red. From it can be calculated for each kidney the amounts excreted, concentration ratios and plasma clearances. Our determinations indicate that less than 10 per cent of phenol red in rabbit's plasma at the concentrations which obtained in this experiment is filtrable.

Table 3 contains data derived from the six experiments, in some of which the excretion of other substances than phenol red was compared with that of inulin. The results are expressed as ratios between the rates of excretion by the two kidneys after the aortic clamp had been released.

The results agree with expectations based upon the earlier work of others and upon that described in the preceding sections of this paper. After the normal circulation and urine formation had been reëstablished in the left kidney, the excretion of water, inulin, urea, and skiodan[6] by the left kidney was conspicuously less than their excretion by the right.

TABLE 3

Ratios of rates of excretion $\frac{left\ kidney}{right}$

	EXPERIMENT 39		EXPERIMENT 40		EXPERIMENT 44		EXPERIMENT 47		EXPERIMENT 48	EXPERIMENT 49
Urine	0.50	0.75	0.47	0.60	0.53	0.59	0.32	0.35	0.24	0.55
Phenol red	1.54	1.96	1.93	0.99	1.77	1.00				
Diodrast-I					3.61	1.06				
Hippuran-I							0.94	1.52		1.44
Inulin	0.26	0.70	0.33	0.55	0.87	0.67	0.14	0.54	0.31	0.78
Urea-N			0.28	0.60						
Creatinine					0.94	0.78				
Skiodan-I									0.39	

This means that no significant quantity of any one of them accumulated in excretable form in the left kidney during the low pressure period. The lower rates of their excretion by the left kidney represent the damage which resulted from the restriction of its circulation.

In striking contrast is the behavior of phenol red, diodrast and hippuran. Despite injury from lessened circulation, the left kidney excreted these in quantities as great as or greater than did the uninjured right kidney. The figures for phenol red, for diodrast, and for hippuran (expt. 49) constitute evidence that a deposition of these substances occurred within the left kidney during the low pressure period and that this deposit was excreted in the urine formed after the normal circulation was reëstablished.

In this group of experiments, as in those described in the previous sec-

[6] Skiodan is monoiodo-methane-sodium sulphonate (52 per cent of iodine). Its plasma clearance in dogs is somewhat less than that of creatinine (5).

tions, the difference between the behavior of inulin and that of several substances, known to be secreted by the mammalian tubule has been so striking as to force the conclusion that the tubules of the dog's and rabbit's kidney are not capable of excreting it.

If this is conceded it seems permissible, in considering this last group of experiments, to use the inulin plasma clearance values as equivalent to glomerular filtration rates and from these to calculate the secretion rates of the other substances which were injected simultaneously with inulin. Plasma concentrations were obtained from curves similar to those which can be drawn on the chart shown in figure 2. Phenol red in rabbit's plasma was uniformly assumed to be filtrable to the extent of 10 per cent of its plasma concentration. The other substances are regarded as wholly filtrable. Table 4 contains the results of such calculations from the

TABLE 4

	EXPERIMENT NUMBER	TIME	RIGHT KIDNEY		LEFT KIDNEY		DIFFERENCE RIGHT FROM LEFT
			Filtered	Secreted	Filtered	Secreted	
		*min.**	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
Phenol red	39	11	0.10	1.00	0.08	1.86	0.86
Phenol red	40	10½	0.10	1.37	0.05	1.87	0.50
Hippuran-I	47	13	2.69	3.01	0.86	5.74	2.73
Hippuran-I	49	6	2.58	2.62	1.97	5.53	2.91
Urea-N	40	10½	5.08	−1.30†	2.93	−0.76†	
Skiodan-I	48	9	5.91	−1.56†	1.81	−0.12†	

* These figures represent the entire time of urine collection after the aortic clamp was released.

† Reabsorbed.

amounts excreted by both kidneys in 5 of the experiments after the aortic clamp was released.[7]

The figures in the last column, obtained by subtracting the amounts secreted by the right kidney from those secreted by the left, involve the assumption that the left kidney after the low pressure period secreted as much from the blood as did the right. If correction could be made for the after effect of the restriction of the circulation in the left kidney on the secreting mechanisms in it these differences would doubtless be greater.

[7] Experiment 44 is not included in the table because the curve of the rate of inulin excretion was distorted at the time of the first urine collection after release of the aortic clamp, presumably because of an injection of saline at the beginning of the period. This circumstance, however, does not alter the significance of the differences between the amounts of diodrast which the two kidneys excreted. Whether or not correction is made for this distortion, such calculations as those used in making table 5 show that the left kidney during the low pressure period was secreting diodrast at about one-third the rate of the right kidney.

They give therefore minimal representations of amounts of substance deposited while filtration was in abeyance. By dividing these figures by the number of minutes between the injection of the substances tested and the release of the aortic clamp we obtain figures for the rates at which the secretable substances were secreted by the left kidney from the blood. From inulin plasma clearances, rates of secretion of the same substances by the right kidney during portions of the same period have been calculated. The comparisons are given in table 5.

The left kidney, in which we believe no filtration was occurring, was from one-fifth to two-fifths as effective in secreting phenol red as was the right, in which filtration was vigorous; for hippuran, it was from one-third to two-thirds as effective.

TABLE 5

	EXPERIMENT NUMBER	RIGHT KIDNEY		LEFT KIDNEY		RATIO LEFT/RIGHT
		Time	Secretion rate	Time	Secretion rate	
		*min.**	*mgm. per min.*	*min.**	*mgm. per min.*	
Phenol red	39	5	0.22	11	0.08	0.36
	40	5	0.25	10½	0.05	0.20
Hippuran-I	47	4	0.65	13½	0.20	0.32
	49	12	0.37	13	0.22	0.60

* The figures in this column represent clearance periods in which the time of blood sampling coincided with the mid-point of the urine collection. For example, in experiment 39, the clearance is that of period R 7–8 (see fig. 2).

These comparisons indicate that the experimental conditions had not interfered with tubular processes of secretion to such an extent as to invalidate the conclusion that inulin is not secreted by the tubules.

The figures in table 4 give no evidence of any secretion of urea or skiodan; the amounts excreted are less than those calculated to have been contained in the glomerular filtrate. This agrees with the results of clearance measurements in intact animals.

Another item of interest appears in these experiments which concerns the rapidity with which the kidney rids the body of these various substances after their intravenous injection. This depends not only upon rate of renal excretion but also upon rate of diffusion from the blood into the tissues. In one experiment 303 mgm. of inulin were injected in 1 minute. One kidney only was functioning. In 16 minutes from the beginning of injection, 33.5 cc. of urine containing 211 mgm. of inulin were collected, 69 per cent of the injected dose. Forty-eight per cent was contained in the urine of 5 minutes of that period.

The following tabulation shows the excretory performance of the right kidney only in experiment 44:

	Inulin	*Phenol red*	*Diodrast*	*Creatinine*
Injected (2 min.) mgm.	560	12	35	60
Excreted (in 6 min. from beginning of injection) mgm.	241	1.5	16.4	15
Percentage excreted	43	12.5	47	25
Relative plasma clearances during the 7 succeeding minutes	1	1.03	3.16	1.35

We have reason to believe that inulin diffuses out of the blood more slowly than any of the other substances. For this reason, despite the conviction that filtration is the only renal process concerned in its excretion, it was eliminated almost as rapidly as is diodrast, which is excreted both by filtration and secretion; more rapidly than phenol red which not only diffuses readily into the tissues but in the excretion of which glomerular filtration is a minor factor.

SUMMARY

In attempts to discover evidence of tubular excretion of inulin, four series of experiments have been made. In the first inulin was perfused through the renal portal vessels of the frog's kidney; none gained access to the lumen unless the tubule had been subjected to injury. Inulin is not secreted by the frog's tubule.

Three groups of experiments were made with dogs and rabbits in which filtration in the kidney was temporarily abolished by lowering the renal blood pressure. Inulin, along with other substances known from the work of Marshall and others to be secreted (phenol red, diodrast, hippuran), was contained in the blood circulating through the kidney during this period. Urine, subsequently obtained, contained enough of the secretable substances to prove that secreting power of the tubules had not been abolished; it contained so little inulin as to warrant the conclusion that the tubules in dogs and rabbits are not capable of secreting that substance. The experiments therefore strengthen our belief that the glomerulus is the sole pathway of excretion of inulin in normal animals.

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PROPERTIES OF THE NERVE FIBERS OF SLOWEST CONDUCTION IN THE FROG[1]

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The expression "fibers of slowest conduction" is used to indicate the group of fibers responsible for the last elevation in the composite electroneurogram; that is, the group called *C* fibers in the original descriptions by Erlanger and Gasser, and by Bishop and Heinbecker, of the occurrence of these fibers in somatic and visceral nerves. In the experiments described in this paper the constants of the fibers have been subjected to further evaluation, and the behavior of the fibers during activity has been examined and brought into correlation with that of fibers of faster conduction.

METHODS. The splanchnic nerve of the bullfrog was selected as the most favorable preparation for the study of *C* fibers, and it was used in all the experiments. Ninety-five per cent of the potential-time area produced by the spikes in the action potential of this nerve is attributable to the group in question (fig. 2). The remaining five per cent of area is distributed among fibers of various velocities. At the head of the series there occurs a small elevation containing about 0.1 per cent of the area of the spikes; it is made up of potentials in fibers that conduct impulses at a maximal velocity of 15 to 20 m.p.s. Then there follows a group in which the maximal velocity is 5 to 7 m.p.s., and in which the slowest velocity is but slightly faster than that of the fibers initiating the large *C* elevation. The latter starts with velocities of 0.5 to 0.6 m.p.s. and contains velocities as low as 0.2 m.p.s. On careful scrutiny, the potentials contributed by the fibers having velocities faster than those of the *C* fibers are discernible in all the records, but no further mention will be made of them here, as they do not interfere with the *C* fiber pictures.

All the experiments have been performed with the aid of D. C. amplification. Various amplifier designs have been employed. The most recent model, with which the records for most of the figures have been obtained, was designed by Doctor Toennies. It is fast enough for nearly

[1] A preliminary report of a portion of this material was presented by C. H. Richards and H. S. Gasser in the Proceedings of the American Physiological Society, 1935.

all purposes, as the reduction is only 20 per cent at 10,000 cycles. Stimulation was effected by break induction shocks or condenser discharges controlled by a thyratron. For tetanic stimulation the thyratron was set into continuous oscillation and the discharges were released from short circuit during the desired periods of activity.

Spike potential. Ordinarily the best method for determining the duration of spikes is to record the impulses in single fibers after they have been conducted to some distance from the stimulus. This method, however, is not completely applicable to *C* fibers, because even in the smallest strand of nerve that it is practicable to prepare, the potentials of single fibers are so small as to fall inside of the noise level. The best that can be done is an approximation to the method by which threshold responses are recorded at high amplification. In these nerves temporal dispersion is large even for short distances. Five millimeters of conduction are sufficient to permit the slowest fibers to lag behind the fastest fibers by more than a full spike duration. Consequently, the conduction distances were kept short—not over 4 or 5 mm., measured from the anode side of the cathode wire to the grid side of the ground wire serving as the active lead. At stimulation strengths near threshold the variation of latency in the setting up of impulses is an additional disturbing factor leading to temporal dispersion of the responses. There is no way of avoiding this error, other than to hold the number of fibers stimulated to a minimum. High amplification must be used and consequently the records are marred by a high representation of noise; and a large series of records must be observed in making a reading on an individual preparation in order to eliminate the effects of random interference.

Despite all the difficulties, a fair value for the duration of the spike can be obtained. An estimate of the homogeneity of the bundle recorded can be made by observing the potential picked up at the second lead. In these small fibers it is almost impossible to obtain a monophasic lead even immediately after a fresh killing of the end; and the ends heal over so rapidly that in the course of a few minutes the size of the potential attributable to the distal electrode is increased. As the longest wave length in *C* fibers (20°C.) is under 5 mm., the phases become well separated in the distance between the leads, and their durations can be measured. For homogeneity in the bundle of fibers under observation the duration of the phases must be alike. Heterogeneity is revealed by a prolongation of the second phase, and in a very sensitive manner, on account of the much longer conduction distance to the second lead. Equality in the duration of the two phases has not been obtained experimentally; but in the best preparations the difference is so small as to indicate that the dispersion in the first phase is slight. In these instances the duration of the spike (*ca.* 20°C.) is between 8 and 9 msec., with the rising phase occupy-

ing about 3 msec. (fig. 1). In less favorable instances the duration is longer.

That the above mentioned durations are not far from being correct is indicated by the measurements of the period of absolute refractoriness. Recovery would be expected to start as in the fast fibers (Adrian, 1921), near the base of the falling phase of the spike; and the measurements of the absolutely refractory period that have been obtained in the best preparations indicate that refractoriness lasts between 7.5 and 8.5 msec.

After-potential. Following the spike, the potential drops abruptly to a positive level (fig. 3*a*) as was originally shown by Bishop, and restoration to the normal occurs only after the positive after-potential has run its course of 1 to 2 sec. Diphasicity, which is usually present, is so readily confused with the positive after-potential that the two are differentiated in figure 2. The preparation had been set up for a monophasic lead, but on standing it had begun to contribute potential to the second electrode. The second phase is well separated from the first and its position can be identified (fig. 2*a*). On rekilling the nerve near the distal lead, the diphasicity disappeared (fig. 2*b*). The notch that remained can best be interpreted as the transition from the spike negativity to a positive after-potential, with an intercurrent small negative after-potential, not large enough to bring the combined potential level to a negative value.

The course of the negative after-potential can most easily be followed as it is developed by veratrine. A few minutes after a one to a million solution of the alkaloid has been applied to the nerve, the level of all parts of the action potential after the spike is found to be more negative than normal. As the poisoning proceeds, the negative after-potential continues to grow, the early part now more rapidly than the later part, so that the negative after-potential comes to dominate the course of the action potential. It comes to a crest close to the spike and then declines (fig. 3*c*).

As the negative after-potential develops it brings into existence along with it a positive potential that succeeds it (fig. 4) and augments and becomes longer in proportion as the negative potential is augmented. At the same time the positive potential, which was normally noted after the spike, is still visible as an indentation on the negative after-potential (at P_1 in fig. 4). These two positive potentials correspond with the first and second positive potentials described for fast (*A*) fibers (Gasser, 1935).

A representative figure for the absolute value of the positive potential after a normal spike would be 0.25 to 0.4 mv. In terms of the spike height after correction for dispersion (as the positive after-potentials sum, while the spikes do so only incompletely), the after-potential amounts to about three per cent. The relationship is in sharp contrast to that obtaining in *A* fibers, in which the positive potential is scarcely measurable after a single spike.

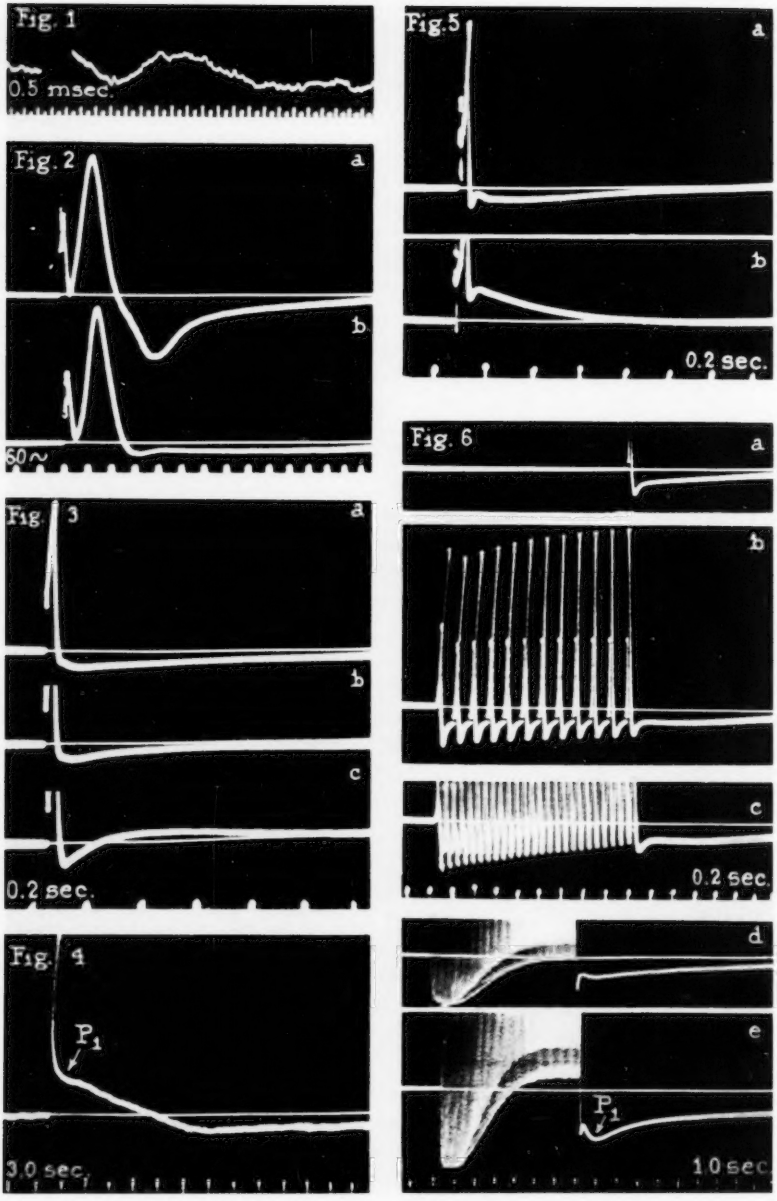

Fig. 1. A threshold *C* response in a strand of the splanchnic nerve of the bullfrog. Conduction distance, 3 mm. Temperature, 18.5°C.

Fig. 2. Maximal action potentials of the splanchnic nerve, *a*, before and *b*, after fresh killing at the distal lead electrode. Ahead of the *C* elevation and fused with

An important point in connection with the observation of the dimensions of the positive after-potential as they have been described is that the nerve must be fresh and rested. If previous to the observation the nerve has been subjected to undue stimulation, it will be conditioned and the negative after-potential will be large enough to overbalance the positive after-potential following the spike. Figure 5 shows how the after-potential is changed from its normal state when a response is evoked during the positive potential following a tetanus. Repeated stimulation in the course of an experiment keeps the nerve in a state analogous to that obtaining after a tetanus, and the negative after-potentials in single responses are abnormally large.

Modification of the after-potential by activity. In *A* fibers, in which the first positive after-potential is small in single responses, the potential is considerably augmented in the course of a tetanus. On the other hand, in *C* fibers, in which the potential is relatively large in single responses, the augmentation during a tetanus is small. Some augmentation must occur, however, as the first positive potential remains clearly identified after a tetanus, despite the development of the negative after-potential which carries over to the production of the second positive potential. In figure 6 *b* the increase in the negative after-potential can be seen in the decrease in positivity that occurs progressively in each successive interval and immediately after the last spike in the train. At the same time the later part of the positive potential after the last spike is larger. Higher frequencies of stimulation cause a further increase in this part of the potential (fig. 6 *c*). When the frequency of stimulation employed for the production of figure 6 *c* is continued for a longer period of time, a well developed after-potential sequence results (fig. 6 *d*). The positive potential is in

the shock escape is the spike of more rapidly conducting fibers. Conduction distance, 4.5 mm. Interpolar distance, 15 mm. Temperature, 19.5°.

Fig. 3. The early course of the development of a negative after-potential, *a*, normal; *b*, 2 min. after painting with 1×10^{-6} veratrine; *c*, 6 min. after veratrine. Conduction distance, 5 mm. Temperature, 18.5°.

Fig. 4. The action potential after complete veratrinization. The crest of the spike is visible at the upper border of the record. At the first thickening of the descending line is the beginning of the negative after-potential, which lasts for 14 sec. and is succeeded by a late positivity. Temperature, 20°.

Fig. 5. After-potentials in a single response set up after a previous tetanus; *a*, response of the rested nerve to a maximal stimulus; *b*, response to a similar stimulus after a 1 min. tetanus at 15/sec. Conduction distance, 4.5 mm. Temperature, 18.5°.

Fig. 6. Modification of the after-potentials by activity; *a*, single response; *b*, 12 responses at 7/sec.; *c*, 26 responses at 15/sec. Both *d* and *c* are at the latter frequency, but on a slower time scale. The amplification in *c* is 2 × that of *a* – *d*. In *b* the full spikes are shown. Conduction distance, 4 mm. Temperature, 19.5°.

two parts, as can be seen in figure 6 *c* which is taken at about twice the amplification used for figure 6*d*. The first part which lasts about two seconds corresponds to the positive potential after a single spike. In its early portion can be seen the negative after-potential augmented because of the tetanus, but now rapidly declining. At its end it passes without abrupt transition into the second positive potential.

Excitability and responsiveness following activity. When the fibers are conditioned by a single response or a tetanus, the curves mapping the ability to respond to supermaximal stimuli in the subsequent period differ from those mapping the level of threshold excitability; and the two functions must be treated separately.

As previously stated, absolute refractoriness ends after 7.5 to 8.5 msec. During the next 20 msec. the size of the responses increases (fig. 7) and at the end of this period the size of the spikes, measured from the positive after-potential level from which they arise to their crests, becomes supernormal. The supernormality lasts throughout the period of the positive after-potential and its course runs parallel with the latter. Thus the shape of the individual curves depends upon the configuration of the attendant after-potential. An extreme variation is found after veratrinization (fig. 8). During the negative after-potential the spike reaches a higher level of negativity than normal; but its height, measured from the level of its origin, is decreased. Nineteen seconds after the start of the first response, when the positive after-potential finally supervenes, the spike is markedly supernormal.

When the spikes are set up in trains, staircasing regularly appears (fig. 6 *b*). Its course depends upon the frequency of the responses. At the higher frequencies, after an initial period of staircasing, the heights of the spikes decrease progressively.

Recovery of threshold. In contrast to the spike height, the excitability does not become supernormal at any time in a conditioning action set up in a normal, rested nerve. At the end of the absolutely refractory period the excitability rises rapidly, but after about 30 msec. have elapsed, recovery is suddenly arrested short of the normal level. From that point onward the excitability approaches the normal over a long flat curve, the time course of which is determined by the positive after-potential. The subnormality has been followed for 1.5 seconds after a single action. In some of the experiments there seems to be a minor peak in the curve at the point of arrest of recovery (fig. 9). This phenomenon probably reflects a transient enhancement of excitability associated with the small negative after-potential seen under favorable conditions in the early part of the positive after-potential (fig. 2 *b*).

When the character of the after-potential is changed by veratrine, the rise of excitability following a single response is continued into a marked

and prolonged supernormal period, and the period of subnormality is delayed. Indirect evidence for the low thresholds obtaining during the supernormal period is found in the spontaneous firing of the fibers that takes place during the course of the negative after-potential (fig. 10).

During the prolonged positive after-potential following a tetanus it has been observed that there is subnormality lasting up to two minutes, but this time cannot be taken as defining the end of the period.

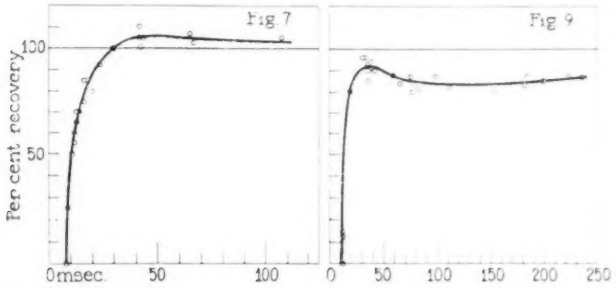

Fig. 7. Recovery of responsiveness in the splanchnic nerve. Both the conditioning and the testing stimuli were about 4 × maximal.

Fig. 9. Recovery of the threshold of excitability.

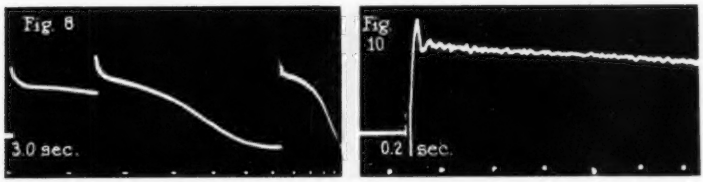

Fig. 8. Dependence of the height of the spike upon the form of the after-potential. Three responses of a veratrinized nerve to maximal break induction shocks. The time scale is logarithmic.

Fig. 10. Response of a veratrinized nerve to a single shock. The normal response of the nerve is shown in figure 2 *b*. Note the spontaneous firing during the large negative after-potential following the spike and its absence before the spike is set up. Conduction distance, 4.5 mm. Temperature, 19.5°.

SUMMARY

All of the components of the action potential that have been recognized in frog *A* fibers can be identified in frog *C* fibers. A spike lasting 8± msec. (see text) is followed immediately by a large positive after-potential lasting 1 to 2 sec. Intercurrent in the trough of the latter there occurs a vestigial negative after-potential. When the nerve has been previously

conditioned by a tetanus, the negative after-potential in a single response is large; and the potential develops to enormous proportions in nerves poisoned with veratrine. It is then followed by a second positive potential; the positive after-potential normally seen in a single action now appears as an incisure in the early part of the negative after-potential.

Tetanization increases both the negative and the positive after-potential. At the end of the tetanus the positive after-potential appears in two parts, of which the first portion corresponds to the potential following a single spike. The size and duration of the second part depend upon the severity of the tetanus.

The absolutely refractory period lasts 7.5 to 8.5 msec. The spike then recovers through a relatively refractory period to a supernormal height, and the supernormality lasts until the positive after-potential is dissipated. In a train of spikes staircasing occurs. The excitability, on the other hand, does not become supernormal. Recovery is arrested short of completion at about 30 msec., and there supervenes a subnormal period lasting as long as the positive after-potential. In modified states in which negative after-potentials develop, supernormal excitability appears.

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PROPERTIES OF MAMMALIAN NERVE FIBERS OF SLOWEST CONDUCTION

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The present studies of mammalian *C* fibers were undertaken in order to define more precisely the constants of these fibers and to extend our information about the aspects of their behavior during activity which have not previously been investigated systematically. All the experiments have been designed with the end in view of learning how the behavior takes place under physiological conditions. Unless special precautions are taken, the manifestations of activity in preparations of isolated nerve may differ widely from what may be expected in the body, particularly with respect to the after-potentials and the excitability cycle. Exact information about the last mentioned features of activity is particularly essential, as an understanding of the properties of the axon, as a sample of the properties of other parts of the neuron, is fundamental to an understanding of the excitation of the neuron at the synapses with preganglionic fibers in autonomic ganglia.

METHODS. The most important consideration when isolated nerves are the subjects of observation is the employment of fresh preparations and the restriction of stimulation to a minimum, as the effects of conditioning are cumulative and long-lasting. Drying is the most troublesome technical difficulty. In order to obviate it, the new nerve chambers have been equipped with a bath into which the nerve may be immersed without opening the box. Krebs' solution in equilibrium with 5 per cent CO_2 and 95 per cent O_2 was used routinely, because of the demonstration by Lehmann (1937) of the importance of maintaining *A* fibers at a normal and constant pH. Actually, as will be seen later, the precaution is of much less importance for *C* fibers, as their sensitivity to changes in reaction is low.

The only test of whether a nerve is being studied under physiological conditions is to compare it with a like nerve under its normal perfusion of blood in the body. Accordingly the method used by Gasser and Grundfest (1936) for mammalian *A* fibers was employed. It depends upon the parallelism between the after-potential and the excitability cycle. Potential and excitability are recorded from the excised nerve, and ex-

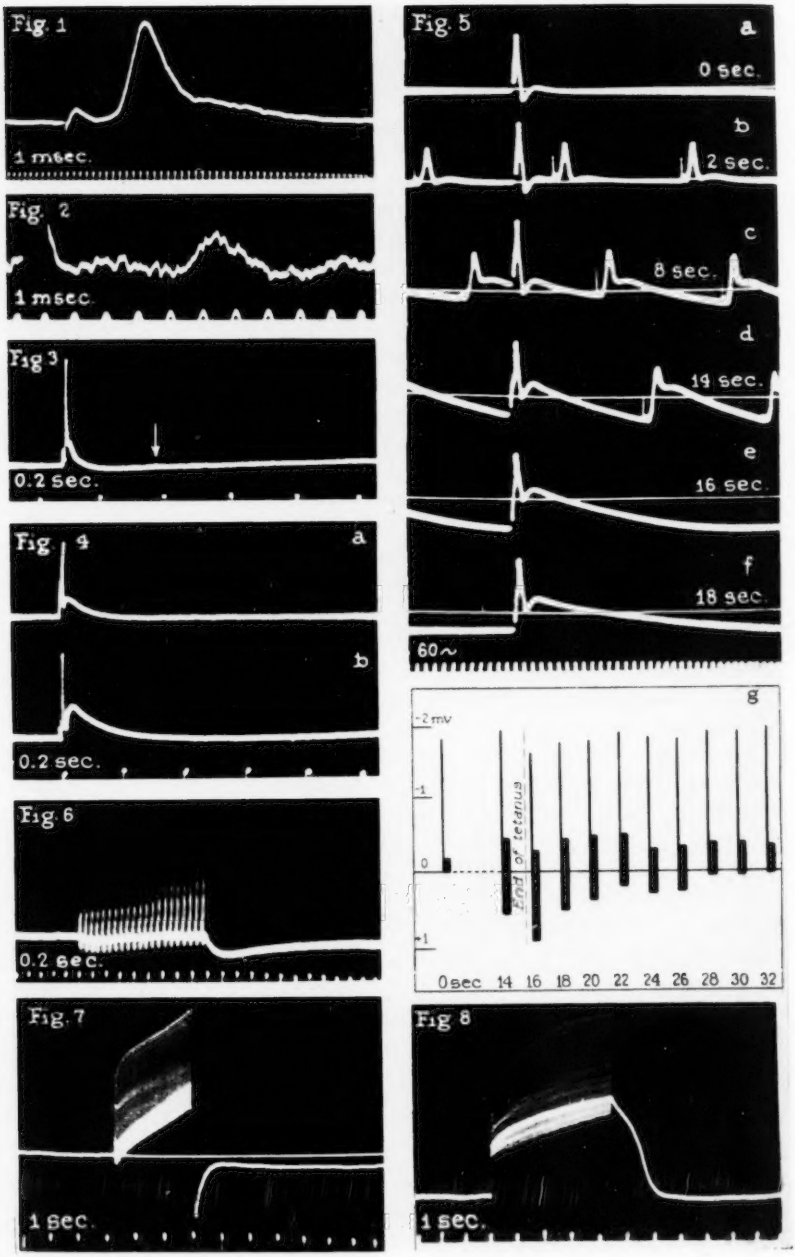


Fig. 1
1 msec.
Fig. 2
1 msec.
Fig 3
0.2 sec.
Fig. 4
a
b
0.2 sec.
Fig. 6
0.2 sec.
Fig. 7
1 sec.
Fig. 5
a
0 sec.
b
2 sec.
c
8 sec.
d
14 sec.
e
16 sec.
f
18 sec.
60~
g
+2 mv
+1
0
-1
End of tetanus
0 sec 14 16 18 20 22 24 26 28 30 32
Fig 8
1 sec.

Figs. 1–8

citability from the nerve *in situ*. If the two excitability curves correspond, it is argued that the after-potential recorded in the excised nerve faithfully represents the one that would be obtained from the nerve in the body. Isolated preparations were made of the hypogastric, splenic, cardiac, and coeliac nerves of the cat, and observations were made of the excitability of the hypogastric and the saphenous nerve (*C* fibers) *in situ*.

In the autonomic nerves mentioned, the *C* elevation comprises either the sole or the major portion of the spike potential. The hypogastric nerve has the largest proportion of fibers with velocities higher than the *C* velocity, but it has been used most frequently because it is a relatively large and unbranched nerve. Its spike record shows the presence of a few fibers with velocities of about 30 to 25 m.p.s. Fibers having velocities ranging between 15 and 3 m.p.s. (the B_2 group of Bishop and Heinbecker, 1930, or the D_1 group of Lloyd, 1937) furnish not more than 10 per cent of the spike potential-time area, and the remaining area is contributed by fibers with velocities between 2.0 and 0.6 m.p.s. (fig. 1). The rise of the chief portion of the *C* elevation starts with fibers conducting at 1.6 m.p.s.

The spike. All the difficulties described in the previous paper in connection with the measurement of the spike duration of frog *C* fibers (Gasser, Richards and Grundfest) hold for mammalian fibers, as do also the same reservations about the correctness of the durations given. In the best preparations of fine strands of nerve, threshold spikes have a duration of about 2 to 2.5 msec. (fig. 2). The absolutely refractory periods as measured on similar preparations last 1.8–2.0 msec.; therefore, 2 msec. may

Fig. 1. Action potential in a hypogastric nerve of the cat. Conduction distance, 10 mm. The small spike ahead of the *C* elevation is caused by fibers conducting at 12 m.p.s. and less. The *C* spike is succeeded by a negative after-potential. The continuation of the action potential into positivity is shown in figure 3.

Fig. 2. A threshold response in a strand from the splenic plexus. The spike, which has an amplitude of about 20 μv, has a duration of 2.7 to 2.8 msec.

Fig. 3. The complete action potential of the nerve illustrated in figure 1.

Fig. 4. Modification of the action potential by changes in pH. Hypogastric nerve, *a*, pH 7.4; *b*, pH 6.8.

Fig. 5. Modification of the action potential during and after a tetanus. Hypogastric nerve, *a*, initial test response; *b* to *g*, during and after the tetanus. The times refer to the start of the tetanus. The thin line found in all the records indicates the resting potential level of the nerve. *g*, graphic representation of the form of the later test responses. The thin vertical lines indicate spikes, the heavy lines negative after-potential. The base line is the resting potential level, as in *a* to *f*. Abscissae, times after start of tetanus.

Fig. 6. Tetanus, same nerve as in figures 1 and 3. The spikes have now become more diphasic. The heavy white lines at the bases of the spikes are the negative after-potentials. They correspond to the heavy black lines in figure 5 *g*.

Fig. 7. Tetanus in a hypogastric nerve of the cat.

Fig. 8. Tetanus at 12/sec. in a veratrinized hypogastric nerve.

be taken as a good figure for the duration of the spikes of the *C* fibers at the head of the series of *C* fiber velocities.

The question may now be raised, whether all the *C* fiber spikes have the same durations (as do mammalian *A* fibers, at least in the range between 110 and 15 m.p.s.), or whether there are durations longer than that holding at the head of the series. A direct answer to this question cannot be obtained, but indirect evidence may be derived from the curve of recovery of responsiveness in the course of an action. The second of a pair of supermaximal shocks separated by 2 to 2.5 spike durations (4–5 msec.) produces a spike, the height of which is 70 to 80 per cent of the first spike (fig. 9). The curve of recovery, except for the time scale, resembles so closely that of the *A* fibers, which represent a homogeneous system, that the inference is strongly supported that the *C* fibers likewise are a homogeneous group. On this basis the wave lengths in the group range between 4 and 1.2 mm.

After-potentials. The *C* action potential is sufficiently similar in all the different nerves studied to permit a generalized description. Following the spike there are well developed after-potentials. Unlike frog *C* fibers, in which the negative after-potential is a scarcely discernible hump in the trough of the positive after-potential, the negative after-potential in mammalian fibers is well developed and at its maximum rises to a position 0.2 to 0.4 mv. to the negative side of the resting level (fig. 3). Following the maximum, the potential decrements at a rate sufficient to permit the action potential curve to pass from negative to positive 50 to 80 msec. after its start. The positive potential reaches its greatest value after 150 to 200 msec. and then decays to become lost to view after 1 to 2 seconds have elapsed.

In the course of the positive potential a faint discontinuity is detectable at about 0.3+ sec., for example, at the point marked by an arrow in figure 3. It is easily missed and its existence might even be doubted, were it not for the fact that it becomes more distinct when observed at the end of a train of spikes. The discontinuity marks the end of the first positive potential, and the only unusual feature in the situation is that it is possible to make out the second positive potential as well as the first in a single action. The large second positive potential is in keeping with the large negative after-potential.

The first positive potential, like the spike, is roughly five times as long as in *A* fibers. It is also relatively larger, but not to the extent that appears in the figures. After 1 cm. of conduction as in figure 3, the spikes are greatly dispersed, while the after-potentials are summed. When the spike height is corrected for dispersion, the positive after-potential is still relatively large, however. It amounts to 1 to 1.5 per cent of the spike height, as compared with 0.2 per cent in *A* fibers.

As a variation of the usual action potential picture, preparations have been encountered in which the negative after-potential component was unusually large, thus causing this phase of the potential to persist for as long as 600 msec. (inset fig. 12). The variation is of significance only when it occurs in freshly excised nerves. For reasons not understood it is usually seen in experiments performed in the late summer or early fall.

C fibers are less sensitive to changes in pH than are *A* fibers, and they are also more resistant to asphyxia (Clark, Hughes, and Gasser). The changes produced, however, are qualitatively according to expectation. For example, the negative after-potential is increased and prolonged at pH 6.8 (fig. 4), and the after-potentials are reduced by asphyxia before the spike is involved.

Modification of the action potential by activity. Tetanization increases both the negative and the positive after-potentials, and spikes set up during a positive after-potential are greatly augmented in size. How these changes take place is shown in figure 5. The experiment was performed with D.C. amplification. No adjustments of the amplifier were made in the course of a set of observations, and the amplifier was sufficiently free of drift to permit the recording of the relation of the potential to the resting level at all times. Two pairs of electrodes were arranged on the nerve for stimulation—*A* at 6 mm. from the lead and *B* at 10 mm. Test responses were evoked from *A*, and *B* was used for tetanization. A normal single testing response is shown in figure 5 *a*. Following this response, the nerve was tetanized for 15 sec. at 4 shocks per sec., and periods of 0.8 sec. out of every two seconds of the tetanus were photographed together with an intercurrent testing response. (The spike of the latter is higher than the component spikes of the tetanus, because of the shorter distance of conduction.) As the tetanus progresses, the negative after-potential as seen immediately after the spikes becomes higher, both with respect to the spike height and absolutely. During the interval between the spikes, however, the negativity declines more rapidly than is normal, owing to the augmentation of the positive potential. Hence the combined potentials become progressively more positive at the moments preceding the advent of the successive spikes. At the end of the train, when newly started actions no longer interfere, the positivity continues to increase rapidly to a maximum. It then declines over a curve the form of which can be seen in figure 5 *g* by following the bases of the heavy lines.

The behavior of the spike and negative after-potential can be followed further in the testing responses evoked from electrode *A*. The increase in the size of the spike and the negative after-potential which started during the tetanus is carried over into the post-tetanic period. Figures 5 *e* and 5 *f* show a response in the first half second after the tetanus and

2 seconds later. In figure 5 *e* the positive potential is still increasing. The spike measured from its level of origin is augmented 40 per cent in height and the negative after-potential is prolonged. Two seconds later (fig. 5 *f*, taken when the positive after-potential from the tetanus has begun to subside again) the excess heights of the spike and negative after-potential have decreased. It is instructive to compare the after-potential periods of figures 5 *e* and 5 *f*, because they illustrate how the contour of a curve is determined by the summation of two potentials. The negative after-potential *appears* to decline more rapidly in figure 5 *e* because it is written on a curve of increasing positivity.

The character of the later testing responses in this series is shown diagrammatically in figure 5 *g*. The potential level of the crests of the spikes tends to hold to a ceiling, but the total spike height decreases as the positive potential from the tetanus decays. Simultaneously the negative after-potentials on the testing responses decrease.

Algebraic summation of the negative and positive after-potentials explains all the variations of the potential form seen during and after a tetanus. If the responses in figure 5 *c* were close enough together to rise out of successive negative after-potentials, it is obvious that the base line would rise. A rise of this sort in the base line is shown in figure 6. The tetanus is at 12 per second. At the beginning of the tetanus the positivity increases more rapidly than does the negativity and the base line falls, but later in the tetanus the negative after-potentials which are increasing, as can be seen in the widening white band at the base of the spikes, dominate the course and the base line rises,—as do also the crests of the spikes. At the end of the tetanus the extent of the tendency to develop positivity when new negative after-potentials no longer interfere is clearly shown.

The same phenomena are shown more strikingly during and after tetani of higher frequency. Figure 7 is taken at 50 per second. At the end of the tetanus the first positive potential (corresponding to the period before the arrow in fig. 3) forms a sharp notch. Beyond the notch can be seen the beginning of the increased and greatly prolonged second positive after-potential. No figure can be given for the duration of the latter because of its mode of ending, but it can usually be traced for 30 sec. to 2 min. The negative after-potential which accumulated during the tetanus is still visible after the first positive potential. As it subsides, the second positive potential becomes larger. Thus the two parts of the positive potential are separated by a maximum of negative after-potential origin.

When the negative after-potential in single responses is large, the configuration of the potentials developed during and after a tetanus follows a modified course. Like frog *C* fibers, mammalian *C* fibers are extraordinarily sensitive to the production of negative after-potential by

veratrine. For example, the negative after-potential may be brought to 85 per cent of the spike height at 10 mm. of conduction (equivalent to about 15 per cent of the spike height corrected for dispersion). During tetanization of a veratrinized nerve the after-potential attains still greater negativity and, as shown in figure 8, the spikes rising above it reach a negativity double that of the crest of the initial spike. The most striking difference, however, is seen at the end of the tetanus. The potential, instead of falling abruptly to form the first positive notch, is continued by a negative potential which obliterates the notch, except for a small inflection in the potential curve near the base of the last spike; and the negative after-potential is continued directly to the second positive potential. A similar ending of the tetanus occurs in the nerves with spontaneously large negative after-potentials which have been described as

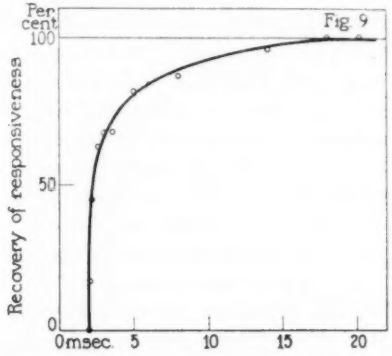


Fig. 9. Recovery of responsiveness in a hypogastric nerve of the cat. Similar curves were obtained with splenic, cardiac, and coeliac nerves.

encountered in the late summer, and in nerves which have been subjected to prolonged survival accompanied by activity.

Modification of responsiveness and excitability by previous activity. Responsiveness is tested by stimulating at various times in the action by a second supermaximal shock. Beginning at the absolutely refractory period (2 msec.), the height of response rises rapidly, but after recovery to 80 per cent of normal is attained, the rate of restoration is retarded and a full height is not reached until 20 msec. have elapsed (fig. 9). During the continuation of the conditioning action no further increase takes place. The spike heights do not become supernormal, as they do in frog fibers or in the later components of a tetanus.

The recovery of excitability was tested *in situ* on the hypogastric nerve in eight experiments made on cats which had been either decerebrated or anesthetized by dial. The hypogastric nerve was approached by an

abdominal incision, but the peritoneum over the nerve was not opened except for the insertion of four fine silver wires to be used as stimulating electrodes. The wires were looped around the nerve without causing injury to the accompanying blood vessels, and the only exposure made of the nerve was at the end, 4 cm. from the ganglion, which was dissected free for the leads. As the animal was kept in a moist chamber at 37°C. and the testing shock was applied at a point different from the conditioning shock, and one supplied with a rich and undisturbed circulation, there was every reason to believe that the excitabilities measured were those of the nerve in a completely normal condition.

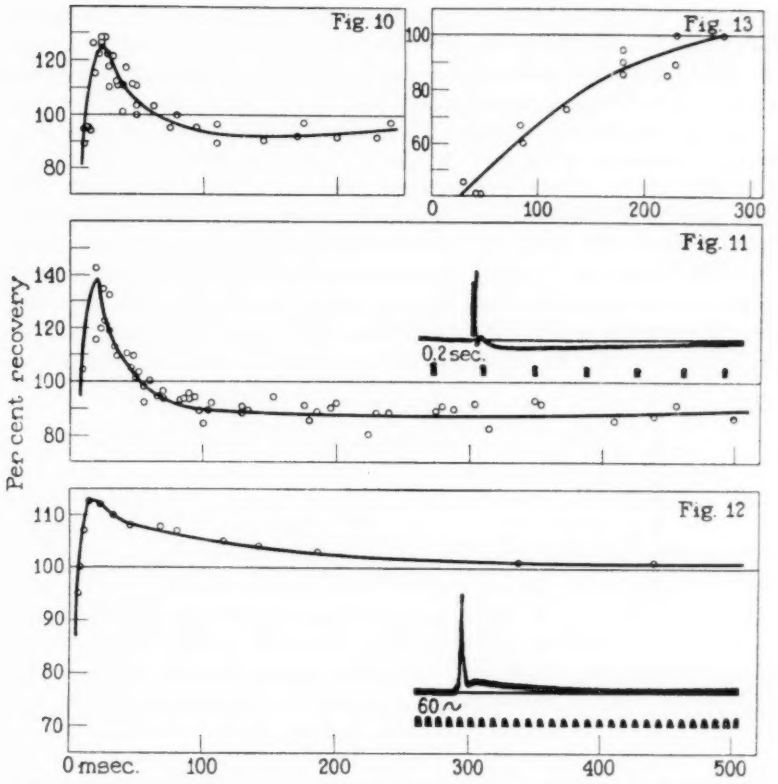
A representative example of one of these excitability curves is given in figure 10. Early points are not included, because the early period of recovery is not a part that is subject to great variation. The threshold returns to normal at 14 msec. Then a period of supernormality appears, with a peak of excitability 28 per cent above normal coming at 25 msec. Supernormality ends at 60 msec. and the ensuing subnormality reaches a maximum at 150 msec. and lasts more than 600 msec.

An excitability curve obtained on an excised nerve is shown in figure 11. In its time dimensions it corresponds to figure 10 and it differs quantitatively only to a slight degree in that the supernormality and subnormality are somewhat larger. The action potential was taken from this nerve and it is included in the figure as an inset. By the test of the form of the corresponding excitability curve the configuration of the potential must be considered as being normal.

In nerves characterized by a long negative after-potential in a single action there is also a long supernormal period. Figure 12 shows a supernormal period lasting about 600 msec. For comparison the action potential of the nerve on which the measurement was made is shown in an inset. Because of their sporadic occurrence there has been no opportunity to ascertain whether nerves of this type would have the same excitability curves in the body.

Mapping of recovery following conditioning by tetanic activity was done by the approximation method used by Gasser and Grundfest (1936). After tetani of 2 to 10 sec. duration, weak testing shocks were applied every two seconds. As was to be expected from the behavior of the after-potentials following a tetanus, the testing response remained subnormal as long as 40 sec.

Excitability curve of the saphenous nerve in situ. Two pairs of stimulating electrodes, insulated except at the tip, were introduced through small slits in the fascial covering of the nerve in the thigh. The slits were 1.5 cm. apart, and the electrodes when in place were insulated from tissues other than the nerve by a small strip of rubber dam. Care was taken not to injure the circulation. After the electrodes had been inserted



Figs. 10, 11, 12, 13. Recovery of excitability in *C* fibers following a single conditioning action.

Fig. 10. Hypogastric nerve of the cat *in situ*. February 23, 1937. The strength of the testing shock was kept constant at a low level. Changes in the height of the conditioned response as compared with the height of the test in isolation gave an index of the excitability.

Fig. 11. Excised hypogastric nerve. March 29, 1937. Inset: form of the action potential.

Fig. 12. Excised cardiac nerve. November 7, 1936. The strength of the testing stimulus was varied until the height of the conditioned test response was made equal to the height of the test in isolation. The strength of shock necessary to produce a constant height of response thus measured the threshold for a group of fibers. Inset: form of the action potential.

Fig. 13. Recovery of excitability in the *C* fibers of the saphenous nerve *in situ*. November 23, 1936.

the skin was closed about them, and a portion of the nerve well below the knee was dissected clear for recording the effects of the testing shocks. In order to eliminate disturbances by reflex movements, the nerve was blocked intra-abdominally by a ligature.

The strong shocks necessary to stimulate C fibers often produce long-lasting repetition of response by the A fibers, and consequently the excitability curve of C fibers could be mapped out only approximately. No supernormality was observed, but there was a relatively deep subnormality lasting 250 to 350 msec. (fig. 13). From the excitability curve it would be inferred that the C fibers in the saphenous nerve are normally without negative after-potential. Its absence would account for the fact that the subnormality does not last longer than the duration of the first positive potential and that the subnormality has an early maximum. The reasons

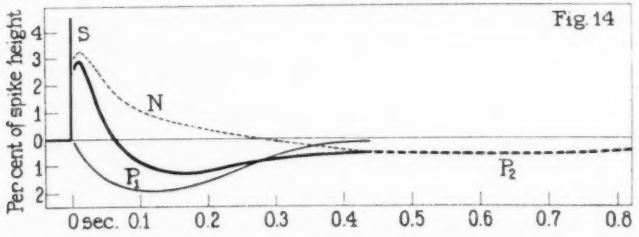


Fig. 14. Schematic composition of the C action potential. The after-potentials are drawn in proper relationship to the spike height. Only the base of the spike is shown.

for the difference between the saphenous and the hypogastric nerves are not known.

Discussion. The detailed study of the various aspects of the manifestations of activity of C fibers has brought out the fact that qualitatively the behavior of C fibers completely parallels that of A fibers. The same events occur, but on a prolonged time scale and with some differences in intensity.

An interpretation of these events in terms of the generalized schema previously presented by Gasser (1937) is attempted in connection with figure 14. A spike (S) lasting 2 msec. is succeeded by a positive after-potential (P_1) lasting about 0.4 sec. It is also succeeded by a negative after-potential (N) which is variable in duration, but which eventually terminates in a positive potential (P_2). The algebraic sum of these components yields the action potential. In A fibers the corresponding durations are: for S, 0.4 msec.; and for P_1, 60 msec. In C fibers, P_1 is proportionately larger in terms of the spike height than it is in A fibers; but

considering the duration of the potential, a relatively higher frequency of tetanization is necessary to bring about its augmentation. No means other than a rapidly repeated train of spikes are known for increasing its size. When augmented, it is not prolonged.

The negative component N can be augmented by a number of means. When it is large, the trough caused by P_1 may be so counterbalanced in a single response that it appears simply as an indentation in N. N is increased by a tetanus and it is then held to be responsible for the increased and prolonged P_2. Likewise in conditions in which N is large in single responses, it is succeeded by an increased or at least a prolonged P_2.

In frog C fibers N is normally very small and a single response consists essentially of S and P_1. During a tetanus N develops and it can readily be identified in the early portions of the successive P_1's.

SUMMARY

Mammalian C fibers were studied in isolated preparations which yielded the same curve of excitability that is attained from the fibers *in situ*. On the basis of this criterion, the nerves were considered to be in a physiological state.

The best figure for the duration of the spike is taken to be 2 msec. In the action potential of autonomic C fibers the spike is followed by a well developed negative after-potential lasting 50 to 80 msec., and the latter in turn is followed by a positive after-potential traceable for 1 to 2 sec. The positive potential reaches a magnitude equivalent to 1.5 per cent of the spike height (compare 0.2 per cent for A fibers), and it is divided into two components of which the first takes up 0.3 to 0.4 sec. After a tetanus the first component is increased more than is the second, but it is not prolonged. The second component is both increased and prolonged in relation to the duration of the tetanus and the frequency of stimulation. During a tetanus both the negative and the positive after-potentials increase in size, and staircasing of the spikes occurs.

The absolutely refractory period lasts 1.8 to 2.0 msec.; 80 per cent recovery of the spike height occurs in 4 msec., but 20 msec. are necessary for complete recovery. As tested after a single conditioning action on the hypogastric nerve *in situ*, the excitability is found to return to normal at 14 msec. Supernormality follows with a maximum at 25 msec. and gives way to subnormality at 60 msec. Subnormality reaches a maximum at 150 msec. and lasts over 600 msec. This sequence fits with the potentials and the excitability cycles recorded in isolated preparations. After a tetanus, subnormality is prolonged, in keeping with the prolonged positive after-potential.

Excitability curves obtained from C fibers in the saphenous nerve *in situ* show no supernormality, and the subnormality is not longer than

would be expected if the positive after-potential contained the first component only.

Variations in the after-potentials and the excitability cycles are described in modified states of the nerves and in one condition which appears to be a physiological deviation of unknown origin.

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SOME EFFECTS OF THYMUS REMOVAL IN CHICKENS

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The thymus gland was known to the early anatomists; Haller, in 1761 described it as a "huge gland scarcely smaller than the kidney." Since his time, up to the very present, its function has baffled the physiologist and clinician.

Soli (1), in 1911, reported that hens, after thymus removal, laid eggs without shells. Riddle (2), in 1924, fed ox thymus to pigeons which were producing eggs deficient in "egg envelope" (shell and white) and reported that their eggs soon after became normal in structure. In this article, Riddle also suggested that removal of the thymus might bring about "deficient or abnormal calcification." He was quite emphatic in his belief that "the thymus substance is indispensable to the production of normal egg envelope." His data seemed "to demonstrate the presence in the thymus of a substance having a highly specific action on the oviduct of birds." Later, in 1931, Riddle and Krizenecky (3) conducted experiments in which they removed the thymus and *bursa Fabricii* from pigeons about 42 days after hatching. In this study no difference was observed in bone abnormalities, and the females produced eggs whose envelopes were quite normal.

Greenwood and Blythe (4), 1931, performed thymus extirpations in three one-year-old laying hens. These hens began again to produce normal eggs fifteen days after the operation.

Work has been done both in this country and in Europe on first, the effect of thymus extracts on calcium metabolism, and second, the effects of thymus removal on calcium and phosphorus level of the blood, and on bone formation. The results are conflicting. Glassner and Haas (5) report that the removal of the thymus in cats leads to deficient callus formation, and that such thymectomized animals, when injected with thymus extract show more rapid callus growth and healing of fractures than the normal controls. They also state that these results were confirmed on human material. Wu (6) believes there is evidence for the existence of two substances in the thymus of the calf, one of which has a hormonal regulation of calcium while the other affects serum phosphorus.

Scholtz (7), 1933, found that thymocresin has no definite influence on blood calcium of rabbits and little, if any, on the blood calcium in dogs.

Morgan and Grierson (8), 1930, removed the thymus from chickens of known age and weight and found that there was no effect on growth or egg production. My own studies bear out these conclusions and further show that neither the shell nor inner structure of the egg is changed by thymus removal.

Anatomical relations. The thymus of chickens occupies a position along each side of the neck beginning anteriorly at about the third cervical segment and continuing downward to the thoracic cavity. There are in all approximately 14 distinct and relatively large thymic lobes, usually seven on each side, loosely joined together. We have never found any thymic material in the thoracic cavity. Greenwood and Blythe (4) found, in some of their material, fragments of thymus situated posterior to the thyroid, sometimes closely adherent to it. The thyroid and parathyroid glands are caudal to the thymus in birds; both lie behind the pleura of the thorax. The thyroid is near the common carotid and the origin of the vertebral artery. The parathyroids are more caudal. In removing the most distal lobe of the thymus the thyroid or even the parathyroids might be inadvertently removed. In case of excision of the latter calcium metabolism would be disturbed, a fact which is well known. Blood calcium would be lowered, bone development might cease, and there would likely be a loss of ability to produce eggs with normal shells.

The thymus gland is especially sensitive to the physical condition of the animal. Malnutrition, starvation, and wasting diseases cause a rapid reduction in its size, bringing about temporary involution of the thymus. In earlier work, with chickens, we have noted a definite reduction in size of the thymus in severe rickets. Since vitamin D deficiency causes rickets with disturbance of calcium metabolism, retarded growth, and bone deformities, it seems probable that, if total thymus ablation also interferes with calcium metabolism, the effects of the two disturbances, simultaneously induced, might summate in producing an earlier and more intensified vitamin D deficiency.

PRESENT PROBLEM AND METHODS. The present studies are concerned with two problems: 1, the relationship of the thymus gland to calcium metabolism, and 2, the effect of removal upon certain phases of reproduction: namely, the development of egg envelope, shell, membrane and albumin.

In four experiments a rachitogenic diet[1] was fed to the young chickens,

[1] Fifty-seven per cent yellow cornmeal, 20 per cent wheat middlings, 20 per cent dry skim milk, 2½ per cent steamed ground bone, and ½ per cent salt. This diet, while somewhat limited in some of the food elements, is adequate for growth in young chickens up to the time when their vitamin D reserve is exhausted. It will bring about satisfactory development for an indefinite period provided vitamin D is supplied.

and the onset of disease, its final severity, its effect on growth and mortality, the serum calcium level (in the blood), and bone deformities were studied in thymectomized and control white Leghorn chickens (experiments I, II, and III), and crossbreeds, Rhode Island Red males on Barred Plymouth Rock females (expt. IV).

RESULTS. Results from these four experiments show no earlier development of rickets in the thymectomized chickens than in their controls. Neither is the disease more severe nor the healing slower than in the control chickens.

At 22 weeks of age (expt. III) average blood calcium in the thymectomized birds was 11.3 with a range of 10.3 to 13.0 mgm. per 100 cc. blood serum. In the controls the average calcium was 11.5, range from 10.8 to 12.7.

TABLE 1

Thymus removal in chickens, experiment III showing onset of rickets, average weights, blood calcium and severity of disease

ONSET OF RICKETS	AVERAGE WEIGHTS			BLOOD SERUM Ca AT 22 WEEKS	DEGREE OF RICKETS PRODUCED
	7 weeks	13 weeks	21 weeks		
	grams	*grams*	*grams*	*mgm. per cent*	
Operated, 6 weeks	273	528	931	11.3	Severe
Controls, 6 weeks	313	501	928	11.5	Severe

Table 1 shows comparative average weights, blood calcium, and intensity of rickets found in experiment III. The data obtained in the other three experiments show essentially the same results.

Experiments I and II ended when the chickens were ten to twelve weeks old, after rickets had been induced and cured, and the required data collected. Thymus removals in experiment I were performed at about 12 days of age. In experiment II, the chicks were 5 weeks old.

The two other experiments involving larger numbers of birds have followed the chickens past the first laying period and well past the time of complete maturity.

Experiment III began with 75 one-day-old chicks. The thymus was removed from 20 during the first week and from 20 more during the second week. In order to search for thymus material which might have been missed in the earlier operation, biopsies were performed during the fourteenth week. Early growth was not noticeably retarded as a result of either operation and growth curves of the two groups were parallel for many weeks thereafter. Healing seemed to be complete within a week after operation. Thirty of the thymectomized chickens lived past the stages of severe rickets, but because of marked deformities and a change

from close confinement to range conditions, not all, either of the controls or of the thymectomized birds, survived to the time of reproduction. Production of eggs began in the hens at about 191 days (6½ mo.) of age. Egg production was delayed about 42 days because of retardation due to rickets. However, there was no significant difference in the time when the first eggs were laid by the operated and control birds. At ten months of age there were still living 11 males, 7 thymectomized and 4 controls; and, after 355 days, out of the 8 surviving females, 5 had been thymectomized and 3 were controls. When the male birds were 310 days old they were killed and a gross as well as a histological search made for thymus material. None was found either in the neck or the thorax. No significant differences were found in the size or development of the various organs in these grown cocks. The same studies were made on the females when they were 355 days old. Table 2 shows the results of blood calcium determinations and bone structure in these tests.

One of the thymectomized females was not producing when the experiment ended. Blood serum calcium in this hen was 12 mgm. per cent (per 100 cc. serum) essentially the same as that found in the males and still within the normal range for young chicks which usually test between 10.5 and 12.5. Blood serum calcium for laying hens is always high. The average for the thymectomized hens was 33.5 and in the controls 26.2, both figures falling within the normal range for hens in production. Variation in averages is perhaps due to small number of hens. Careful studies were made of the eggs as to size, shell texture, and thickness; thickness and character of the shell membranes and internal quality. Thickness of the shell and membranes was determined by a spherometer calibrated to measure 1/1000 inch. Internal quality of the egg, white and yolk, was judged according to the standards and charts of Van Wagnen and Wilgus (9) (see fig. 1). These data are given in table 3.

TABLE 2

Blood serum calcium and bone structure in mature chickens

	MALES		FEMALES	
	Controls	Thymectomized	Controls	Thymectomized
Blood serum calcium (averages)	11.9	11.9	26.2	33.5
Bones (x-ray studies)	Normal	Normal	Normal	Normal

In the 164 days from the time the first pullet began production and the end of the experiment, the control pullets gave an average production per cent of 45, not quite one egg every alternate day as compared with 46 per cent for the thymectomized hens. In the average total weight of

eggs to average hen weight the slight advantage was in favor of the controls. In the chart used in judging internal quality of eggs (Fig. 1) 1.0 represents a perfect egg. The yolk is rounded, firm, and of uniform surface color. The thick white is firm and relatively great in amount. It is held close to the yolk and stands up well (0.250 inch or more in depth). Other indications of quality of the white, such as clearness and freedom from blood or meat spots, were observed. There was considerable variation in the quality of the eggs from the same hen, and some hens regularly laid eggs of better quality than those of other hens, but no decided difference was found between the experimental and control groups when averages were taken. The shell and membranes were "normal" and would have been pronounced "good" by the professional egg handler. Measurements of thickness of both shell and shell membranes were practically the same for both thymectomized and control hens.

Experiment IV. In experiment IV we took advantage of a sex-linked color difference which appears in the Rhode Island Red-Barred Plymouth Rock cross. This made it easy to separate the sexes in the newly hatched chicks. We therefore performed thymectomies on about one-half of the females during the first week, and on the remainder when they were ten days old. There were 17 survivors of the first operation. Against these were 9 controls. This experiment ended when the birds were 16 months old and were again at the height of production.

TABLE 3

Comparative data on production and egg quality

	CONTROLS	THYMECTOMIZED
Average number eggs per hen	73.66	75.5
Average weight of eggs to hen weight	2.72	2.31
Average egg quality	2.03	2.39
Average thickness of shell	0.0148 inch	0.0148 inch
Average thickness of membrane	0.0025 inch	0.0023 inch

Up to the age of five months rate of growth was the same in the chicks having the thymus removed and their controls, but after that time the controls became definitely larger. In addition there has been a rather high mortality since maturity. Five of the thymectomized hens died after four months of normal production. Cause of death seemed also to be in the dysfunctioning of the crop and esophagus. In searching for thymus material on post mortem, none was found. None of the controls died.

Eggs from the thymectomized hens were as well developed and as perfect in shell and white as those from controls. There seemed to be absolutely no difference in quality of eggs from the two groups.

Further studies around the question of the inter-relations between the thymus and other glands of internal secretion are in progress.

Discussion. The thymus gland in chickens is found in the neck and its relatively large size makes these animals favorable subjects for study of thymus function. Our earlier studies (10, 11, 12, 13) on calcium metabolism in chickens have supplied valuable data and experience useful in carrying out these later experiments and have facilitated our efforts.

Hoskins (14) and Rowntree (15) in two recent books on endocrinology speak of the probable importance of the thymus gland on the development of the egg envelope in birds and in the control of calcium metabolism. I

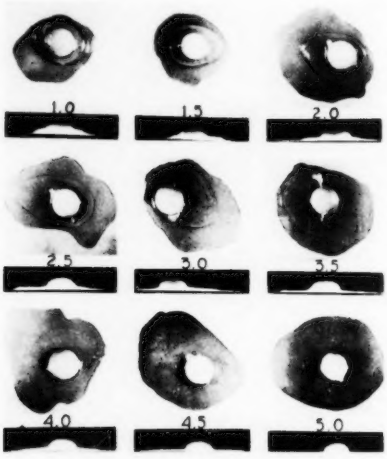

Fig. 1. Showing gradation in quality: 1.0 a perfect egg; 5.0 a fresh egg but with no firm white. This egg ranks lowest in quality. The side view, below each egg, shows the height of the white.

believe that there is little if any justification for the continuance of either of these ideas.

The results of our work and a review of the literature to date fail to reveal any specific endocrine function for the thymus. It seems more doubtful still that there is any specific control of calcium metabolism by this gland.

One cannot neglect, however, the interesting results obtained by Einhorn and Rowntree (16) showing extreme precocious development and early sex maturity as a result of injections of Hanson's thymus extract from calves into successive generations of rats. This work has given a new impetus to the study of the thymus.

SUMMARY

No significant differences have been noted in the rate of growth or the time of maturity of thymectomized and control chickens.

Onset of experimental rickets occurred at exactly the same time in both and the severity of the disease was not more pronounced in the one than in the other. Blood calcium in young chickens, regardless of sex or age, was as high in the thymectomized birds as in the normal controls. X-ray photographs of the tibia and femur bones of the mature chickens showed no bone defects.

Egg production began at the same age in both groups. Careful studies of the eggs reveal no significant differences in egg quality, including thickness, texture and soundness of shell, thickness of shell membranes, and quality and texture of egg white. It seems from these experiments that removal of thymus from young chickens has no effect upon calcium metabolism, or upon the so-called "egg envelope" of the eggs produced after the hens have matured.

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RECOVERY OF BLOOD-PERFUSED MAMMALIAN NERVES[1]

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The effects of activity on the properties of isolated nerve fibers, especially electrical excitability, have been described frequently; but the first experiments on the recovery of blood-perfused nerve fibers were not published until recently (18, 19, 11, 15). The description given by Gasser and Grundfest (11) of the recovery cycle of electrical excitability of nerve *in situ* agrees in general with ours (19). Gasser and Grundfest examined the relation between the electrical excitability of the nerve during the successive phases of recovery and the potential changes (spike and after-potentials) that accompany and follow conduction of the nerve impulse, while we have studied the relations of excitability, conduction rate, and height of response.

Technique. Etherized or decorticated rabbits were used, and by means of a cathode ray oscillograph the responses to induction shocks applied either to the oculomotor or sciatic nerve were recorded from the internal rectus muscle, the nerve to the inferior oblique muscle, the tibialis or gastrocnemius muscle, or the deep peroneal or tibial nerve, according to the nerve stimulated. About 20 experiments were performed with the oculomotor nerve; records were taken chiefly from the internal rectus muscle. More than 50 experiments were performed with the sciatic nerve, with records usually from the deep peroneal nerve. In addition, 6 sciatic-peroneal preparations in cats were used.

The method of making the oculomotor-internal rectus preparation has already been described sufficiently (17). When the nerve to the inferior oblique muscle was used, its peripheral end was carefully laid across electrodes mounted in a chamber in which, with the aid of a vaporizer, the nerve was prevented from drying or cooling. In the sciatic preparation, after the animal had been rendered insensitive, the pelvis and femur

[1] Most of the experiments here reported were carried out in the Central Institute for the Deaf, St. Louis, during 1935–36, and were aided by grants from the Rockefeller Foundation to the Central Institute for the Deaf and to Washington University for research in science.

on the side to be used were fixed by appropriate clamps, and the sciatic nerve was temporarily exposed sufficiently to permit section of the muscle branches and insertion of insulated stimulating electrodes, usually at the level of the trochanter major of the femur and sometimes at other more peripheral points. The insertion of the electrodes and the other necessary operations were accomplished with a minimum of tissue injury and with little, if any, disturbance to the circulation of the nerve. The preparations for recording involved central section of the tibial or peroneal nerve, whichever was not to be used, and insertion of concentric needle electrodes into the appropriate muscle, or exposure at the level of the knee of a sufficient length of peroneal or tibial nerve to permit application of recording electrodes. It was of course not possible to avoid interference with the circulation of this exposed stretch of nerve.

The induction shocks used as stimuli were produced by discharging condensers of about 0.1 mfd. through the primary of a Harvard coil, of which the secondary was shunted by a 1000ω potentiometer. The duration of the shocks never was longer than 60 to 70 μsec.

Late recovery of excitability. For the purposes of the present investigation, recovery of excitability can be measured satisfactorily by the variation of the height of response to a stimulus of constant strength, because in an unconditioned nerve the height of response is within certain limits proportional to the stimulus strength (fig. 1, *E*). The experimental procedure consisted of recording the varying height of response to a constant submaximal stimulus at varying intervals after the conditioning response, and immediately afterwards recording the height of the unconditioned responses to shocks of progressively increasing strength. By subsequent measurement and comparison of heights in the two sets of records, it was possible to determine the strength of shock to which the constant testing stimulus had been made equivalent by the conditioning process. An example of the actual conditioned height readings obtained and the calibration curve by which they were transformed into terms of excitability is given in figure 1, *D*, *D'* and *E*.

The recovery cycle of blood-perfused nerves is by no means uniform. Figure 1 illustrates the four types of recovery found in our experiments. Curve *A*, the most frequent form, includes all phases—refractory, supernormal, subnormal—thus far observed with nerves *in vivo*, and differs in no essential respect from the recovery curves reported by Gasser and Grundfest. Our observations on the time-relations are strikingly similar to theirs: end of relative refractoriness at 2–5 msec., peak of excitability at 4–7 msec., transition from supernormality to subnormality 10–20 msec., maximum subnormality 20–30 msec., return to normal excitability 50–100 msec. However, Gasser and Grundfest found greater degrees of both supernormality and subnormality than we did; their average values (7

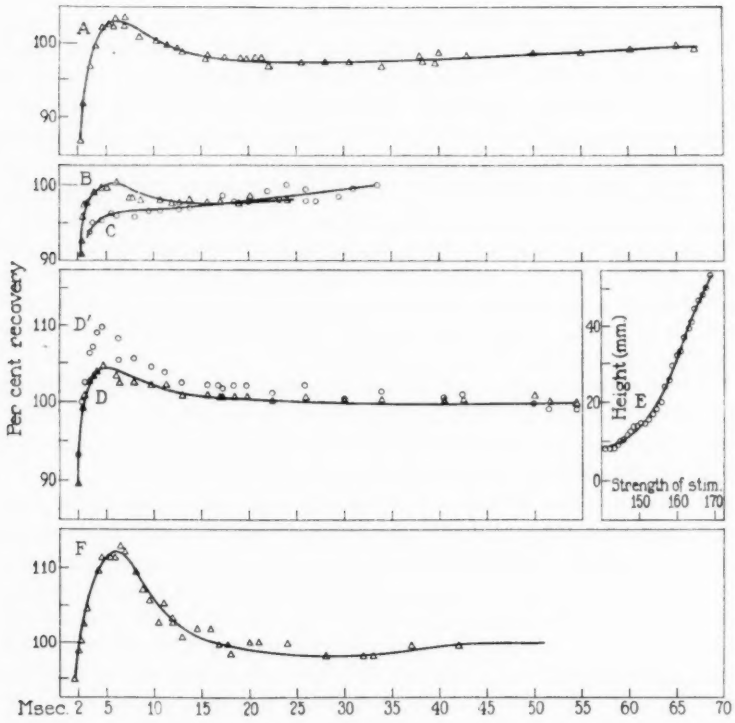

Fig. 1. Recovery of excitability after single conditioning shock. Abscissae: msec. between conditioning stimulus and testing stimulus.

A. Rabbit sciatic-peroneal, light ether narcosis. Conduction distance for conditioning and testing stimuli, 62.5 mm. Observations made 1 hour after preparation begun, ½ hour after first stimulation of nerve. 10/30/35.

B. Rabbit sciatic-peroneal, ether narcosis. Conduction distance, 72 mm. for conditioning stimulus, 30 mm. for testing. Observations made 1¾ hours after preparation begun, 24 min. after first stimulation of nerve. 4/29/36.

C. Same nerve as *B*. Observations made at the start of the experiment.

D'. Rabbit sciatic-peroneal, deep ether narcosis. Actual height readings in millimeters, plotted according to right-hand ordinate scale. Conduction distance for conditioning and testing stimuli, 65 mm. Observations made 50 minutes after preparation begun, 7 minutes after first stimulation. 10/31/35.

D. Height readings plotted in *D'* transformed into terms of excitability by calibration curve *E*.

E. Height of unconditioned response to stimuli of different strengths. Abscissae: potentiometer setting in ohms. 10/31/35.

F. Rabbit oculomotor-internal rectus, ether. Observations made 2 hours after preparation was begun, 1¼ hours after first stimulation. 10/22/35.

per cent supernormality at the peak, 3 per cent subnormality at the nadir) are about twice those calculated from our findings.

In the type of recovery exemplified by curve *B*, the early peak of excitability does not exceed and may not even reach the normal level of excitability. This variation was sometimes recorded, as in the nerve providing the data for curve *B*, after a single conditioning response, and it is the form regularly observed after a series of conditioning responses (Gasser and Grundfest; see also below).

Another type of recovery cycle including only one phase of depressed excitability is exemplified by curve *C*. It is important to note that this curve embodies observations made immediately after the preparation was ready for experimentation and with as little stimulation of the nerve as possible. Shortly thereafter the same preparation yielded the recovery plotted in curve *B*. A recovery of form *C*, obtained at the start of an experiment, always passed rapidly into the *B* or *A* form, and was ordinarily not observed *in toto*, although indications of its fleeting existence were usually found if the preparation was made rapidly, and stimulation prior to observation of recovery at the relevant shock intervals held to a minimum.

Recovery of the form of curve *D*, with a long-lasting phase of supernormal excitability, was observed in a few preparations.[2]

Since attempts to control the form of the recovery curve—except the reduction of the supernormal peak by rhythmic stimulation (see later)—were not successful, only meager evidence was obtained as to the factors determining which form would be manifested. *a*. Supernormality was sometimes decreased by forced hyperventilation of the lungs, and following (although not during) asphyxia by re-breathing, supernormality was in some cases markedly increased. *b*. Form *C* (little or no suggestion of early peak of excitability) was usually not observed with freshly prepared nerves of animals of which other nerves had been under experimentation. *c*. Ether seemed to have no specific effect on the form of recovery; increasing or decreasing the depth of anesthesia did not modify the form of the recovery curve significantly, and the same forms were observed in decorticated as in etherized animals. *d*. Supernormality was not observed in moribund rabbits.

The substitution of the gastrocnemius or tibialis muscle for the peroneal nerve as a recording device, which makes it possible to keep the whole nerve with a normal blood supply, did not alter the general form of the recovery curve. With the oculomotor preparation, the internal rectus muscle provided a recording device so satisfactory that it was but rarely replaced by the nerve to the inferior oblique muscle. The recovery

[2] According to observations made by one of us (Lorente de Nó), the *A* form of recovery changes into the *D* form for a period of several minutes following severe tetanization.

curves recorded with the oculomotor-internal rectus preparation are strikingly similar to the sciatic-peroneal curves, the most conspicuous difference being the lesser development of subnormality in the case of the oculomotor. An oculomotor recovery curve including an exceptionally high degree of supernormality is reproduced in figure 1, *F*; an oculomotor recovery of type *C* has been reported elsewhere (18, fig. 1).

Conduction time and velocity. The term conduction time is here applied in the usual way to the interval between the start of the stimulating shock and the start of the action potential at the recording electrode. This interval includes in addition to the time necessary for conduction of the impulse between the two points, the shock-response time at the stimulated point. As shown by the fundamental observations of Blair and Erlanger (1933) in excised nerves, the fluctuation with occasional long duration of this interval when shocks of barely threshold strength are used, accounts for the observed fluctuation of the conduction time. The fluctuation, and also the variation of spike form observed with threshold shocks, disappear when the shock strength is increased slightly (4, fig. 2; 8, fig. 35). Blood-perfused nerves behave in the same way: shocks barely above threshold strength produce responses after varying times and of varying forms; strengthening of the shock results in stabilization of the shock-response time (fig. 2), and further strengthening stabilizes the spike form also. Exact calculation of conduction velocity on the basis of the stabilized conduction time is however impossible, because the shock-response time at the cathode is not known.

Another cause of error in the calculation of conduction velocity is the fact that the position of the actual stimulating cathode may vary with the strength of shock (so-called spread of stimulus (1)). Fortunately this factor, known not to be important under ordinary circumstances in isolated frog nerve (5, 24), was found to be negligible in most cases in blood-perfused mammalian nerves as well. Control records showed that under our experimental conditions, the shock-response time remained constant for shock strengths from 60 to over 250 per cent of the strength giving a maximal *A* spike. Not until the shock strength was increased to about 4 times "maximal" was there any shortening of shock-response time; with still stronger shocks the surge to ground through the recording lead caused impulses to start at or near this point.

It is evident from the control experiments that submaximal responses of as low as $\frac{1}{3}$ the maximal height give shock-response times that may be used for the calculation of approximate conduction velocities. Shock-response times of two such responses of practically equal height may be compared with an accuracy of about 2 per cent. Observations made in this way indicate that the conduction rate is constant from moment to moment, and also over considerable periods of time, although in the

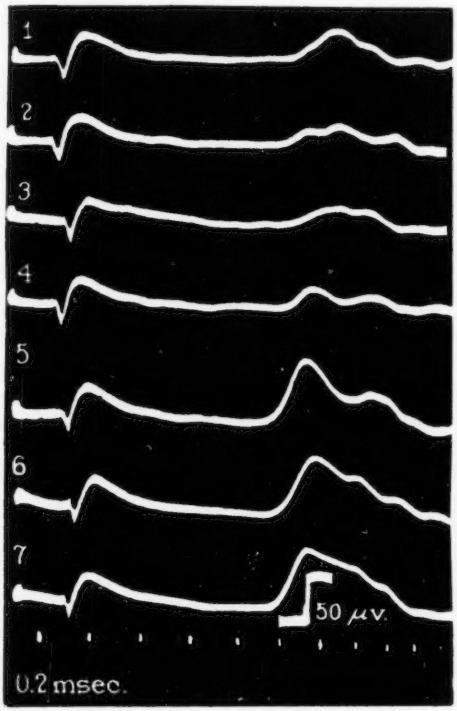

Fig. 2

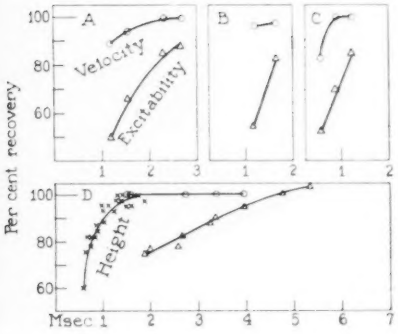

Fig. 3

Fig. 2. Conduction time after weak stimuli. Rabbit sciatic-peroneal. Conduction distance, 81 mm. Responses to single shocks; shock for *5*, *6* and *7* few per cent stronger than for *1*, *2*, *3* and *4*. Owing to differences in latency the individual fibres respond at different intervals after delivery of the shock, and the recorded potentials contain several spikes. With the stronger shock some fibres respond after nearly the minimal latency, but others respond after long latency. 3/15/36.

Fig. 3. Recovery of excitability, height and conduction velocity after one conditioning response. Rabbit sciatic-peroneal, ether. Abscissae for *A*, *B* and *D*: msec. between conditioning and testing stimuli; for *C*: this value minus time required for conditioning response to reach testing electrodes. Conduction distance: *A*, 62 mm. for both stimuli (1/30/36); *B*, 65 mm. for both stimuli (1/29/36); *C*, 65 mm. for conditioning, 20 mm. for testing stimulus (1/29/36); *D*, 55 mm. for both stimuli (12/20/35). Both stimuli hypermaximal for measuring recovery of height; conditioning maximal and testing submaximal for other recoveries.

course of experiments lasting for several hours it generally decreases by a small amount (about 5 per cent in 3 or 4 hours).

In view of recent reports (21, 22) that the conduction rate of a nerve increases when its connection with the nervous system is severed, a number

of experiments were performed in order to measure the amount of the change under our experimental conditions. The separation of the nerve from its central connection was usually accomplished by cutting or crushing the nerve, or by blocking it with heat 10 or more mm. above the anode of the stimulus. In other experiments the nerve was not interrupted, but the brain stem was sectioned caudal to the red nucleus, or the anesthesia was suddenly changed from very light to very deep. Contrary to expectation, no increase of conduction velocity was ever observed. In 4 of the 16 experiments in which the nerve was severed (15 peroneal, 1 tibial) there was a small increase in conduction time for which no apparent cause was found. It may have been due to decrease in conduction rate, but it may also have been due to change in the nerve near the interrupted point.

Because of the many possible causes of error, quantitative values for the conduction rate of even the fastest fibers must be stated with reservation. The usual assumption of a uniform rate throughout the length of nerve used is not applicable, since despite all possible experimental precautions the peripheral stretch of nerve exposed for recording is not in the same condition as the rest of the nerve. The velocity of conduction in the portion of nerve having a normal blood supply, between two widely separated stimulated points, was calculated from the difference between the conduction times from the two points to the recording electrodes; the mean value found was 104 meters per second, and values up to 140 meters per second were obtained.

Recovery of velocity of conduction and height of response in their relation to recovery of excitability. Study of the early part of the relatively refractory period is fraught with difficulty, in large part due to the strength of shock necessary to elicit responses, but the difficulty is increased in multifibered nerves by the differences in properties of the individual fibers. In these nerves a deficit of response may be due to absence of response in some fibers, to subnormal height of response, or to both; for this reason many of the problems concerning nerve recovery can be solved only in preparations of single fibers. Multifibered nerves provide satisfactory data on several points however, particularly after recovery has progressed somewhat.

In addition to these difficulties, the chief obstacle to determining the early recovery of the speed of conduction, for the fastest fibers at least, is that the speed of the conditioned response increases as it is conducted along the nerve. The longest conduction times observed in our experiments (immediately after the absolutely refractory period and for conduction distances down to 8 mm.) have been about twice the unconditioned time; however, in view of the rapid increase in velocity at the start of recovery (cf. fig. 5), it is not possible to make a quantitative statement as to the initial speed of the conditioned response.

Our experiments indicate that in blood-perfused nerves, height of maximal response as well as speed of conduction was depressed during the early relatively refractory period; but as is evident from figure 3, both height and conduction rate recovered much more promptly than electrical excitability did, and this despite the fact that the segment of nerve near the recording electrode was not blood-perfused. Moreover, the height and the speed of conduction did not manifest the changes (supernormality, subnormality) shown by excitability during the later part of the recovery cycle. Figure 3, *D* presents in chart form the data obtained in one nerve in which recovery of excitability and that of height of response were recorded for the later part of the recovery. In this nerve, the completeness of the recovery of conduction rate and the persistence of the depression of excitability were established at the interval (about 1.5 msec.) showing complete recovery of height. Parts *A*, *B*, and *C* of the figure present data from two other nerves and show the promptness of recovery of conduction rate as contrasted with that of excitability. For purposes of comparison with recovery of excitability, recovery of conduction time has been plotted as recovery of conduction velocity, on the incorrect but here permissible assumption that the conditioned impulse travels at a constant velocity along the nerve. The contrast between the recovery of conduction rate and that of excitability is marked, whether the testing stimulus is applied at the same point as the conditioning or at a different one, although the recovery of excitability is more prompt in the latter case (cf. fig. 3, *B* and *C*). At a given interval the degree of recovery of excitability varied considerably in different nerves, but the recovery of velocity as well as that of height was always more advanced at all intervals, and was usually complete when the excitability was 70–80 per cent of its resting value.

The validity of the comparison of the three recovery curves—excitability, height, velocity of conduction—might be questioned because of the use of different sets of fibers for the three curves; but the experimental findings dispose of this objection. For recovery of height, all the *A* fibers are used, and for that of conduction rate only the fastest portion of these, while for recovery of excitability an intermediate set of fibers is the critical one. If the difference in the fibers used affected the result, it would tend to diminish the observed divergence between the recovery of excitability and that of height, since the recovery of height is more rapid. Furthermore, since the spike form becomes normal when the conduction rate of the fastest fibers recovers, all the *A* fibers evidently recover their rate at practically the same time.

Due apparently to the strong stimulation applied to the nerve for measurements during the relatively refractory period, supernormal excitability was rarely observed in nerves in which the refractory period was studied (an exception to this is shown in fig. 3, *D*); but conduction time during the

supernormal period was recorded in a number of other nerves. Excitability in these nerves usually rose during the supernormal period to 105–107.5 per cent, and reached 115 per cent in one nerve at a certain shock interval; nevertheless, it was never possible to demonstrate any change of conduction time.

Similarly, during the subnormal period the conduction rate was found to be normal within the limits of error of measurement (1–2 per cent). Records were made not only during subnormality following single conditioning shocks, when the slight change in excitability would make the expected change in conduction time near the limit of experimental observation, but also during the relatively great subnormality obtainable by repetitive stimulation (cf. below).

Recovery after rhythmic activity. The numerous published reports on the effects of activity on nerve fibers include reports of prolongation of the absolutely refractory period (Woronzow, 26), depression of height of response (Forbes and Rice, 9) and slowing of conduction (Gerard and Marshall, 12; Woronzow, 26; Gasser, 10; Titeca, 25). Depression of excitability is always found. Since most of the published work on height of response and conduction rate is based on amounts of activity far beyond the physiological range determined by Adrian and Bronk (3; see also Adrian, 2), recovery of these functions and of excitability as well was examined after varying amounts of conditioning activity. Our findings on the late recovery of excitability after moderate activity agree qualitatively with those of Gasser and Grundfest, and will therefore not be reported at length. The chief difference observed, greater decrease of the early peak of excitability with relatively little increase of subnormality (fig. 4 *A*), may be related to the fact that in general the frequency of the series of conditioning responses was somewhat greater, and the number of responses fewer, than in the experiments of Gasser and Grundfest. Despite the high frequencies used in the conditioning series, there were two or three preparations in which actual supernormality occurred after repetitive stimulation, and was indeed no lower than in recovery after one conditioning response. One of the marked cases of this phenomenon happened to occur in a cat and is shown in figure 4*B*. It is evident that in this experiment the recovery curve after one response was of the exceptional *D* type (see footnote, p. 329).

The ease with which the latter part of the recovery of excitability is delayed by moderate rhythmic activity (summation of subnormality) is in great contrast to the relative stability of the first part of the recovery (up to perhaps 80 per cent), and to the stability of the recovery of conduction rate. In studying conduction rate during the subnormal period, it was found that with repetitive series of 5 to 25 shocks at frequencies of 333 to 685 per second, the excitability was easily depressed to 80 to 90

per cent (in one case to 60 per cent) of normal for intervals of 5 to 20 msec. after the end of the conditioning series; but the conduction time did not change to a measurable degree (1 to 2 per cent). (The conduction distance for the testing responses varied from 10 to 64 mm., and the conditioning series was applied 13 to 45 mm. central to the testing stimulus.) The contrast between recovery of excitability and velocity is presented graphically in figure 5, which shows a recovery of conduction rate no faster after a single conditioning response than when this response was preceded by

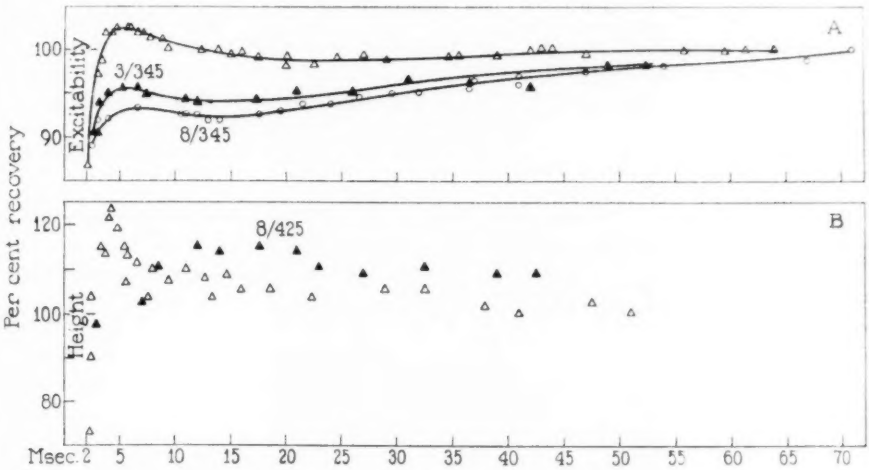

Fig. 4. Effect of repetitive conditioning on recovery of excitability. Abscissae: msec. between latest conditioning stimulus and testing stimulus. △, recovery after 1 conditioning response; ▲, ○, recovery after stated number of conditioning responses at frequency indicated, e.g., 8 responses at 345 per second. *A*. Rabbit sciatic-peroneal, ether. Conduction distance 68 mm. for conditioning stimuli, 24 mm. for testing stimulus. 4/24/36. *B*. Cat sciatic-peroneal, decortication followed by very slight etherization. Conduction distance, 39 mm. for conditioning stimuli, 25 mm. for testing. Height of submaximal testing response recorded as percentage of unconditioned height. 5/10/36.

five responses at the rhythm of 500 per second. The recovery of excitability is however decidedly faster in the former case.

Recovery of height is as stable as recovery of conduction rate; this is illustrated by figure 6. The experiment from which these records were taken was one of a number so arranged that two hypermaximal shocks (conditioning and testing) could be delivered through one pair of electrodes, while through another pair more centrally located a series of maximal shocks of any desired frequency and duration could be delivered at different

intervals preceding the conditioning shock. The effect of the rhythmic series on recovery was determined by examining for each interval between the two single shocks the response to the testing shock when preceded by the conditioning shock only, and when preceded by the series and the conditioning shock. All these experiments showed that with short series of responses at high frequency preceding the conditioning shock, the recovery of height of the testing response, and of its speed of conduction as well, was practically as fast as when the refractoriness was created by the conditioning shock alone. The records reproduced were taken from an experiment in which the series consisted of 19 shocks at a frequency of 500 per second, and ended 8.3 msec. before the single conditioning shock. A series of 34 shocks at this frequency and interval was also without apparent effect on the recovery of height. Moreover, the duration of

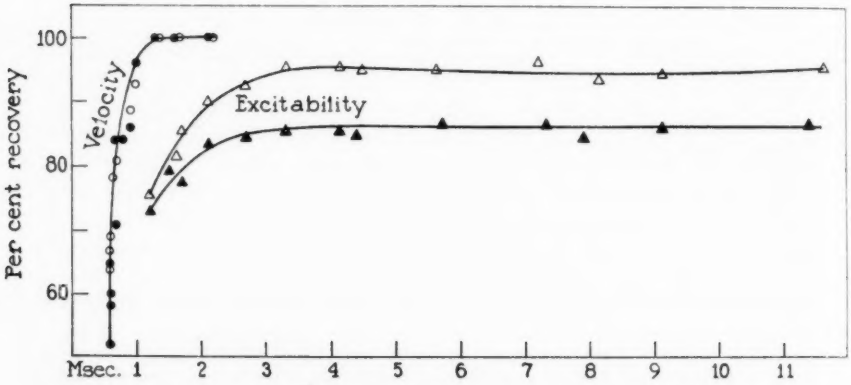

Fig. 5. Recovery of excitability and conduction velocity. Rabbit sciatic-peroneal. Abscissae: msec. between testing stimulus and latest conditioning stimulus. Conduction distances: 19 mm. for testing and latest conditioning stimuli; 70 mm. for earlier conditioning stimuli. △, ○, recovery of excitability and conduction velocity after 1 conditioning response; ▲, ●, recovery after same conditioning response following by 2.8 msec. a series of 5 stimuli at 500 per second. 2/29/36.

the absolutely refractory period was the same after such activity as after a single response (0.5 msec. or somewhat less).

In order to determine the amount of rhythmic activity necessary to bring about the depression of conduction rate described in the literature, experiments were performed with conditioning series running 5 to 100 seconds at frequencies of 300 to 775 responses per second. The testing shock was applied $1\frac{1}{2}$ to 2 seconds after the end of the series, and at 2 second intervals thereafter. In these experiments definite slowing of conduction was not observed in nerves stimulated 300 to 600 times per second, but

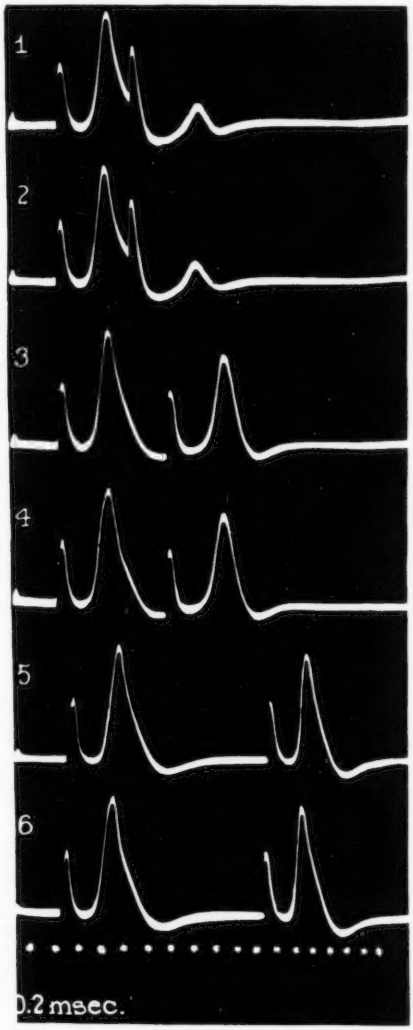

Fig. 6. Recovery of height and conduction rate after single conditioning shock (records 1, 3, 5) and after this shock preceded by a series of 19 shocks at 500 per second (records 2, 4, 6). Interval between last shock of series and single conditioning shock, 8.3 msec. The shocks of the series were maximal, the two others hypermaximal. Rabbit sciatic-peroneal. 2/29/36.

was clear in other nerves stimulated 775 times per second for 20 to 70 seconds. It was of course greatest at the time the first testing shock was applied, and decreased gradually, to disappear in 10 to 20 seconds.

Discussion. In regard to the recovery of excitability *per se*, only the supernormal period need be discussed. Gasser and Grundfest (11) consistently observed actual supernormality after a single response, and are therefore inclined to believe that this would characterize recovery after a single response under normal conditions. Our opinion that normal recovery after a single response would be rather of type *C* is based on the fact that this type of recovery was found when the preparation was made with the smallest amount of manipulation and delay, and the nerve had been submitted to but little stimulation; recovery with supernormality (type *A* or *B*, fig. 1) was not found until after more experimentation.

Whether or not actual supernormality occurs after a single action under normal conditions is however not a matter of great importance, since the normal function of nerve is to carry successions of impulses; our findings on recovery after repetitive stimulation agree closely with those of Gasser and Grundfest.

On the other hand, the question of whether the process underlying supernormality (and therefore the negative after-potential (11 and other references there)) is a physiological one, is of considerable importance; and on this point also there is entire agreement. Since the oculomotor-internal rectus preparation, in which the nerve is in no way disturbed, may manifest supernormality to a testing shock applied to the nerve after a conditioning response produced by trans-synaptic stimulation as well as by electrical stimulation of the nerve (20, figs. 2 and 3), there can be no question that supernormality, and therefore the process underlying it, may be induced in nerve by the passage of an impulse under entirely physiological conditions.

The great variability of the late part of the recovery cycle may be ascribed as least in part to the difficulty of keeping the preparation under constant metabolic conditions. The effects of hyperventilation and asphyxia mentioned above may be recalled in connection with the work of Schmitt and Gasser (23) on the relation between oxygen consumption and after-potential. Recent work by Lehmann (16) has shown that ionic factors likewise affect the after-potentials. The great modifications that he has shown to be produced by slight changes in pH are of particular interest in this connection, since Dusser de Barenne, McCulloch and Nims (7) have shown that such changes actually take place in the body, even in organs as generously irrigated as the cerebral cortex.

The contrast between the variable late part of the recovery cycle, and the earlier part during which 1, the recovery of excitability seems to be relatively constant, and 2, the recovery of height and conduction rate is

completed (except after activity far exceeding the physiological range), suggests that the division between these two parts at 1.5–2 msec.[3] may mark an event of some importance in the physiology of nerve.

The usual absence of changes in conduction rate in mammalian nerves during the late part of the recovery cycle is remarkable in view of the published findings in isolated frog nerve (13, 14, 10; cf., however, Cooper 6). At present no satisfactory explanation of these divergences can be offered.

Our observations indicate that the speed of conduction of a nerve does not increase when its connections with the central nervous system are severed. They are, therefore, in apparent contradiction to the observations of Monnier and Jasper, and of Monnier. Monnier and Jasper used shocks very near threshold and observed considerable variations in the conduction time (cf. fig. 2 above). When this observation was originally made, the existence of variable latency in nerve response to short induction shocks (4) was not generally recognized, and consequently the only possible explanation of variable conduction time was in terms of variable speed of conduction. Now however the observations of Monnier and Jasper may be taken as proof that latency is also manifested by nerves *in situ*; this we confirm. The concept of subordination of the speed of conduction to the state of the cells of origin must be abandoned.

SUMMARY

1. The recovery of rabbit and cat sciatic nerve *in vivo* after one or more conditioning responses has been recorded from the tibialis or gastrocnemius muscle or deep peroneal nerve. The *in vivo* recovery of the rabbit oculomotor nerve has been recorded from the internal rectus muscle or the nerve to the inferior oblique muscle.

2. The recovery of excitability, presumably because the metabolic condition of the preparation cannot be maintained constant, varies considerably in different preparations, and even in the same preparation, during the course of the experiment. Four typical forms of recovery cycle are illustrated in figure 1. There are reasons to assume that type *C* is the normal one, although type *A*, which includes a peak of supernormal excitability between the refractory period and the subnormal period, is more often recorded.

3. Recovery of conduction velocity occurs more promptly than that of excitability; there are no late changes in velocity corresponding to the supernormal and subnormal periods of excitability. The recovery of height conforms to that of velocity.

4. Rhythmic activity within physiological limits does not affect the

[3] Woronzow's division of the refractory process (26) into two parts is not identical with the division suggested here, since his point of division falls within the absolutely refractory period.

recovery of height and velocity, nor that of excitability early in the relatively refractory period, but greatly delays the later part of the recovery of excitability.

5. These experimental findings suggest the division of the recovery cycle into two parts, during the first of which (1.5–2 msec.) height and velocity recover completely, and excitability recovers to an extent (roughly 80 per cent) that does not change significantly after rhythmic activity. The completion of the recovery of excitability during the second part of the cycle is greatly affected by rhythmic activity and other conditions.

6. The conduction rate of the fastest fibers in the sciatic-peroneal preparation is constant from moment to moment and over long periods of time. It is not affected by separating the fibers from their central connections.

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QUANTITATIVE RELATIONSHIPS OF CALCIUM AND CEPHALIN IN EXPERIMENTAL THROMBIN FORMATION

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A standardized technique has been developed for the quantitative study of the factors involved in thrombin formation *in vitro* (4). The reagents are controlled for "purity" and stability. They include 1, calcium salts; 2, chemically pure cephalin (from brain); 3, prothrombin (poor in cephalin: from platelet-free plasma), and 4, fibrinogen (prothrombin free). Controlled physical variables include *a*, dilution (total); *b*, concentration (individual reagents); *c*, pH, and *d*, temperature. Routine thrombic mixtures consist of 5 cc. prothrombin solution (controlled for stability and, therefore, uniform for each series of experiments) *plus* 0.5 cc. cephalin solution *plus* 0.5 cc. $CaCl_2$ solution. The "age" of the mixture is timed from the moment of adding the calcium salt. Clotting-tests are made at 38°C. on 0.5 cc. samples of thrombic mixture *plus* 1.0 cc. of fibrinogen, the clotting-time being recorded at the onset of definite (visible) fibrin formation. The experimental unit is the prothrombin activation (thrombin formation) curve obtained by plotting clotting-times against age of thrombic mixture.

Figure 1 illustrates the use of the method and the influence of the temperature factor. Note the evidence of *dynamic* changes proceeding in the thrombic mixture from the moment of adding the calcium salt. Thrombin formation is slow at first but the rate of the reaction accelerates until a maximum is reached as indicated by the shortest clotting-time obtainable with the particular mixture under investigation. At 15° 20°C. the maximum is maintained for some hours, indicating a stable thrombin. At 38°C. the maximum is really an optimum since it is succeeded by a regressive phase. A considerable degree of activity may be restored to the deteriorating thrombin by alkali and acid treatment (cited 5). Hence the degradation may be regarded as a process of metathrombin formation, though not in Howell's sense of the term. At 15°C. the maximal activity is developed much more slowly but the final potency is greater than the optimum at 38°C. It may be concluded, therefore, that a small amount

of metathrombin formation occurs *pari passu* with the thrombin production at the higher temperature.

Figure 2 represents the effect of limiting (minimal) cephalin concentrations in a series of thrombic mixtures in which the prothrombin and calcium factors are kept constant. These particular curves are incomplete since the maxima are not included, but they do show a uniform shifting of the curves (particularly affecting the *rate* of thrombin formation) with very small decrements in cephalin concentration. Expressed in terms of dry weight of added cephalin in the routine thrombic mixtures (v. supra), it is evident that in the range of 0–10γ[1] of cephalin, it is entirely

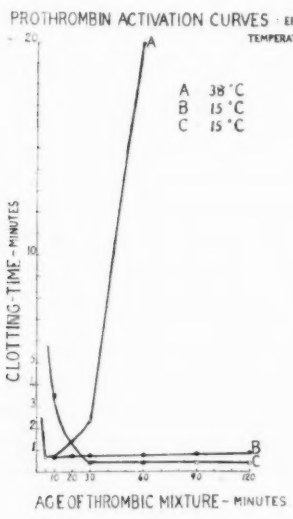


Fig. 1

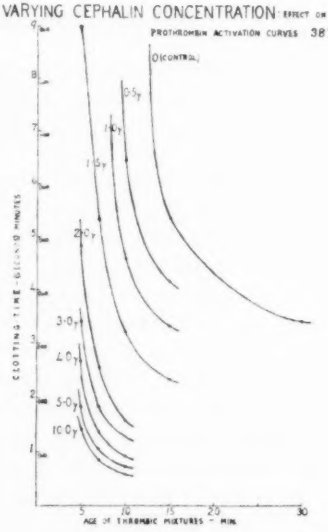


Fig. 2

possible to detect with satisfactory accuracy variations of 0.0005 mgm. The practicability of using the present system as a means of estimating the specific coagulant phospholipids will not be discussed further at this time since a simpler modification of the "clotting-test method" is being studied with this end in view.

In a number of complete experiments (at 38°C.) it was found that the maximum thrombin level was a little weaker with each decrease in cephalin concentration. The establishment of this point demands the most careful scrutiny of the experimental conditions with particular reference to the stability of the weaker thrombins, which, as has been shown, is largely

[1] $1\gamma = 0.001$ mgm.

a function of the temperature. Table 1 offers data obtained with the utmost technical care, including a low incubation temperature (15°C.) at which the thrombins are adequately controlled for stability. Note the persistent differences between the maximal activity (shortest clotting-times) of the several thrombins of varying cephalin concentration. There appears to be no escape from the conclusion that the phospholipid concentration determines not merely the rate of thrombin formation but also the *amount* of thrombin produced. That the differences between the maxima are due to the amounts of prothrombin activated is demonstrated by the addition of stronger cephalin in the later tests. The increase in activity thus obtained (to an approximately constant value of 12″)

TABLE 1 •

Effects of varying cephalin concentration on clotting-power developed in thrombic mixtures

Each mixture consists of 10 cc. prothrombin solution + 1 cc. N/10 $CaCl_2$ + 1 cc. cephalin (cited dilutions) incubated at 15°C. Clotting-times (seconds) at 38°C.: 0.5 cc. thrombin + 1.0 cc. prothrombin-free fibrinogen solution.

CEPHALIN DILUTION	5 MINUTES	30 MINUTES	1 HOUR	2 HOURS	3 HOURS	4 HOURS†	5 HOURS†	6 HOURS† (INCUBATED AT 15°C.)
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*
1:1,000*	20	7	7	7	7	10 (12)	11 (12)	12 (13)
1:10,000	23	10	9	9	10	12 (12)	12 (12)	13 (13)
1:100,000	90	23	18	15	15	18 (12)	19 (17)	20 (18)
1:1,000,000	720	140	50	30	30	36 (12)	38 (13)	(14)
1:10,000,000	2,160	680	330	73	65	74 (12)	78 (12)	90 (12)

* A 1:250 cephalin gave an optimum C.T. = 8″.

† In parentheses, C.T. of test samples which were incubated at 38°C. for 5 min. with 1/5 vol. of 1:1000 cephalin (to convert unchanged prothrombin to thrombin) before adding fibrinogen.

shows the presence of unchanged prothrombin in the weaker mixtures, but not in those with sufficient amount of cephalin at the start. It is concluded that the cephalin is used up during thrombin formation, the inference being that it has quantitatively combined with the prothrombin (and calcium).

In table 2, especially in the second series, the cephalin is increased to the point of "excess." A definite inhibitory effect is apparent at the higher cephalin concentrations, although in no case was the phospholipid present in amounts chemically equivalent to the calcium content (cf. 9).

The degree of inhibition appeared to bear some relationship to pH since the most marked effects occurred in experiments in which phenol-red indicator demonstrated a definite acidity in the clotting mixture. Small amounts of N/100 HCl produced a similar inhibition. The anti-coagulant

TABLE 2

Effects of varying both cephalin and calcium concentrations upon the development of clotting-power in thrombic mixtures, incubated at 38°C.

First series: 5 cc. prothrombin + 0.5 cc. N/40 $CaCl_2$ + 1:1000 cephalin (amounts varied from 0.05 to 4.0 cc.) + distilled water (to make up constant total volume = 10 cc.).

Second series: Similar except for use of N/10 $CaCl_2$.

Clotting-time (seconds): 0.5 cc. thrombin + 1.0 cc. fibrinogen (prothrombin-free) at 38°C.

	$CaCl_2$ SOLUTION	CEPHALIN (1:1000)	Ca: CEPHALIN RATIO (APPROXIMATE EQUIVALENTS*)	INCUBATION PERIOD OF THROMBIC MIXTURES (AT 38°C.)							
				2 minutes	5 minutes	10 minutes	20 minutes	30 minutes	45 minutes	60 minutes	120 minutes
	cc.	*cc.*		*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	
1	0.5 (N/40)	0.05	175:1								4½ hours
2		0.5	17.5:1				2,550	735	370	300	810 seconds
3		2.0	4.4:1		Overnight	1,540	275	190	210	360	
4		4.0	2.2:1		Overnight	1,520	320	240	255	420	
5	0.5 (N/10)	0.05	700:1	940	105	80	97	143		350	
6		0.5	70:1	380	70	55	100	138		420	
7		1.0	35:1	328	65	72	126	185		600	
8		2.0	17.5:1	580	80	72	130	213		1,155	
9		4.0	8.8:1	2,040	155	97	190	287		1,800	

* Calculated on basis of molecular weight of cephalin = 700.

TABLE 3

Effect of diminishing proportions of calcium salt on development of thrombic activity

Mixtures consisting of 5 cc. prothrombin solution + 0.5 cc. 1:1000 cephalin + 0.5 cc. calcium (cited strengths), incubated at 38°C. Clotting-times (seconds) for prothrombin-free fibrinogen (38°C).

Ca CONCENTRATION	2 MINUTES	5 MINUTES	10 MINUTES	20 MINUTES	30 MINUTES	60 MINUTES (INCUBATED AT 38°C.)
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*
N/100*	Trace	Trace	Trace	Trace	Trace	Trace
N/50	4500	1050	230	45	30	45
N/10	15	7	10	14	22	53

* Trace of clots in all tubes, overnight.

action of complex organic acids recently noted by Wadsworth, Maltaner and Maltaner (8) may also be recalled. It is, therefore, suggested that the inhibitory phenomenon is due to the "acidifying" effect of cephalin (especially in the presence of calcium salts—v. *infra*).

Table 3 shows the effect of diminishing the proportion of calcium salt,

keeping the prothrombin and cephalin constant. Note the rapid falling off in potency as the Ca-ion concentration is decreased. Notwithstanding the fact that the thrombins were incubated at 38°C. and, therefore, subject to some metathrombin formation, it is probable, in view of the similar phenomena with cephalin, that minimal amounts of calcium determine amount as well as rate of thrombin formation.

Table 4 demonstrates an inhibitory influence of "excess" of calcium. We have no explanation of this fact (ref. 3).

Possible rôle of calcium-cephalinate. The question arises whether the qualitatively similar effects of varying the calcium or the cephalin concentrations independently may be attributable to any quantitative chemical relationship between these two reagents. Following the work of Levene and his colleagues (who confirmed the original suggestion of Thudichum, 1884), it has been customary (ref. 5) to emphasize the differences between various phosphatides, first, on the basis of the nitrogenous base and,

TABLE 4

Effect of increasing quantities of calcium salt on development of thrombic activity. Same mixtures and conditions as in table 3

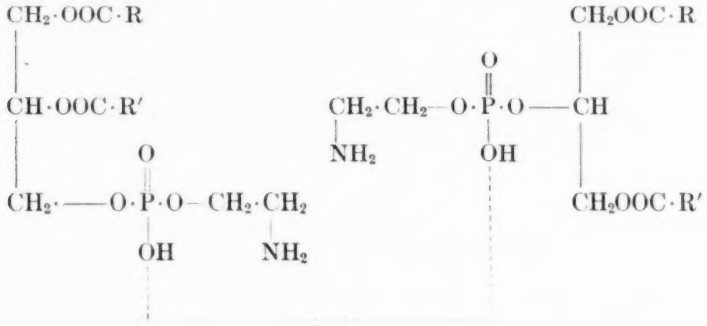
Ca CONCENTRATION	30 SECONDS	1 MINUTE	2 MINUTES	5 MINUTES	10 MINUTES	20 MINUTES	30 MINUTES	60 MINUTES	90 MINUTES	120 MINUTES (INCUBATED AT 38°C.)
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*
N/10	170	110	30	7	10	18	33	75	137	170
N/5		65	22	10	15	37	65	115		
N/2.5		83	45	20	25	50	70	125		
2N	1,510	940	950	830	745	630	610	370	337	355

secondly, on the varying degree of unsaturation of the fatty acids in the diglyceride portion of the molecule. It has only recently been pointed out that the co-existence of acidic and basic groups in the phosphatide molecules makes possible two important alternatives in their physicochemical behaviour. They may be dissociated in aqueous solution and function as ampholytes *or* they may exist in the form of non-reactive "internal anhydrides."[2] As Wadsworth, Maltaner, and Maltaner (7) have clearly pointed out, this may very well explain the specificity of certain phospholipids for blood coagulation and other processes. It would certainly explain why other phospholipids than cephalin may evince coagulant properties and why some true cephalins (e.g., the synthetic distearyl cephalin of Grün and Limpächer) are inactive.

It is the *dissociable* phosphatides which react with salts, as their consti-

[2] A somewhat modified explanation of the same phenomena may be given in terms of a *Zwitterion* theory (T. H. Jukes: *J. B. C.* **107**: 783, 1934).

tution (that of a substituted phosphoric acid) would indicate. Calcium is peculiar in forming salts which are either insoluble in aqueous solution (as we have observed with a preparation made by the method of Maltaner) or are merely depressed as regards calcium-ionization, yet remain in colloidal solution (the more usual behaviour with most of our brain cephalins). The chemical reaction may be illustrated by the following graphic equation in which, purely for the purpose of illustration, the two reacting cephalins are depicted in the α and β forms[3] respectively (*N.B.* R and R′ denote the fatty acid nuclei):



$$CaCl_2 \rightarrow Ca(Ceph)_2 + 2HCl.$$

The free acidity change in this reaction may be strikingly demonstrated by the use of brom-thymol-blue indicator. On adding the blue-colored calcium salt solution to the greenish cephalin, a visible shift to the yellow is immediately observed. The subsequent degree of turbidity or precipitation will depend upon the colloidal conditions which, as previously noted, vary with different phospholipid preparations.

Wadsworth, Maltaner, and Maltaner (7) were unable to induce coagulation with their precipitated calcium-cephalinate (washed free from acid), but could restore it to the active form by an acid-extraction technique. We have confirmed.

In the experiments of table 2 we note particularly tests *2* and *6*. These have the same prothrombin and cephalin content. There is a much greater thrombin development in *6* which had four times as much calcium. If the prothrombin activation were controlled by an intermediary of the nature of calcium-cephalinate (formed according to the cited equation) then, since the calcium is in excess in both tests, the thrombin formation

[3] According to J. J. Rae (Biochem. J. **28**: 152, 1934) the brain phosphatides occur mainly in the α-form.

should be solely a function of the cephalin concentration, i.e., the same in the two cases. The facts distinctly oppose this possibility. They show, on the other hand, a decided dependence of thrombin formation upon the calcium concentration with the cephalin playing only a minor rôle. It is true, nevertheless, that for any given calcium concentration the rate and amount of thrombin formation are secondarily influenced by the cephalin concentration.

Since both the cephalin and the calcium are operating in the same direction to improve thrombic potency it is not surprising that the coagulant activity bears no relationship to mere equivalence of the Ca : Cephalin ratio (e.g., tests *2* and *8*).

Conclusion. The present data indicate that the primary determinant of the rate and to a slight but significant extent the amount of thrombin formation *from a fixed quantity of prothrombin* is the concentration of calcium ions. The fact that for any particular calcium concentration the activity developed is in turn controlled by the cephalin content is adequate evidence that the cephalin must act by binding the calcium to the prothrombin. This corroborates the finding of Mills (6) and Ferguson (1) that prothrombin cannot be activated by calcium alone in the absence of "available" cephalin.

There is no evidence that the reactions, quantitative in general character though they be, follow any known stoichiometric laws. Until the isolation of the prothrombic factor as a definite physico-chemical entity—instead of the empiric material with which we are still forced to experiment—this means only that we can definitely exclude any simple calcium salt of the phospholipid from the rôle of intermediary, notwithstanding the specific nature of such compounds. May we not draw the inference that the calcium-containing "intermediary complex" in thrombin formation (evidence for the existence of which has been offered in a previous communication (2)) is a colloidal complex of *all three* known components, viz. prothrombin + calcium + cephalin?

SUMMARY

Using a standardized and carefully controlled technique, quantitative data have been obtained concerning the effect upon the rate and degree of thrombin formation (in vitro) of varying the cephalin and calcium content of experimental thrombic mixtures. Both these factors are essential for thrombin formation in the system studied. Diminution of either results in a progressive lessening both in the rate of thrombin formation and to a lesser, but equally significant extent, in the amount of thrombin produced. Excess of either is inhibitory.

When both calcium and cephalin are varied in parallel series of experiments, it becomes evident that the calcium is the more fundamental

determinant of the thrombin-formation from any given sample of prothrombin. The cephalin exerts an important secondary influence, since increasing amounts (up to a limit, determined in all probability by pH conditions) successively improve the thrombin formation.

It is concluded that the specific compound which calcium can form with cephalin is not a direct controlling factor but that the "intermediary substance" in thrombin formation must be a colloidal complex of all three precursors, viz., prothrombin *plus* cephalin *plus* calcium.

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PROTHROMBIN IN CHICKENS

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In previous communications concerning hemorrhagic diathesis in chicks produced by vitamin K deficiency, it has been shown that the most probable explanation of the low clotting power of the blood is a decrease in the amount of prothrombin (1, 2). Although it has been possible to precipitate prothrombin from plasma of normal chicks by means of the acetone method of Howell, and the acetic acid method of Mellanby, corresponding precipitates obtained from the plasma of K-avitaminous chicks are inactive (3).

The fact that it has not been possible to precipitate active prothrombin from the plasma of vitamin K-deficient animals might be explained in two ways; either the prothrombin is absent, or it is present in an inactive form, i.e., in a form which is activated more slowly by tissue factor and calcium ions than is normal prothrombin. In the present investigation evidence is presented which indicates that the plasma of vitamin K-deficient birds does not contain prothrombin in an inactive form. Furthermore it is shown that the small amounts of prothrombin which occur in pathological plasma can be determined quantitatively and related to the prothrombin content of normal plasma.

By employing prothrombin deficient plasma as a reagent, a new method of approaching certain problems concerning the nature of prothrombin has been made. Thus it is possible to show that prothrombin is not identical with complement, and in addition it is possible to study the species specificity of prothrombin. The chemical nature of prothrombin is as yet not clear, but evidence which now is available in the literature indicates that prothrombin is associated with the pseudoglobulin fraction of the plasma proteins.

METHODS. White leghorn chicks were used in all experiments. On the eighth day of life the feeding of the vitamin K-deficient diet was begun. The diet was composed of casein (Harris lab., highest purity) 20 per cent, yeast (yeast foam powder) 15 per cent, sucrose 62.3 per cent, salt mixture 2.7 per cent ($CaCO_3$:2.00; $MgCO_3$:0.10; ferric ammonium citrate:0.16; NaCl:0.44; KI:0.000025). To 100 parts of the diet were then added 4 parts cod liver oil. This diet is practically identical with that used in earlier ex-

periments (4). The control fowls were fed an adequate diet composed of Spratts, growing mash 80 per cent, dried fish meal 15 per cent and dried spinach 5 per cent. Chicks fed the deficient diet developed hemorrhagic phenomena in 2 to 4 weeks.

The clotting of fowl blood will be delayed if drawn through a needle inserted in a large vessel. In all experiments blood was drawn into ice cold, paraffined tubes and then centrifuged. Clotting determinations were made by the method of Fischer (5). Extracts of chick embryo or of chick brain were used as the clotting agent; such extracts are stable for several hours. When a definite amount of tissue factor is added to plasma the clotting time will depend upon the prothrombin concentration, if all other conditions are the same. In each Fischer tube is placed 0.2 ml. plasma to which one drop of the clotting agent is added, and the clotting time recorded with a stop watch. All determinations were carried out in a waterbath at 37°C.

Is prothrombin present in vitamin K-deficient chicks in an inactive form? In order to ascertain whether the plasma of vitamin K deficient animals contains prothrombin in an inactive form the following experiments were performed.

a. Normal plasma was mixed with an equal volume of Ringer's solution.

b. K-avitaminous plasma was mixed with an equal volume of Ringer's solution.

c. K-avitaminous plasma was mixed with an equal volume of Ringer's solution containing 2 per cent normal plasma.

Immediately after mixing the above solutions were placed in a waterbath at 37°C. From time to time samples were removed and the clotting time determined. The results are given in table 1.

From the results given in table 1 it can be seen that when a small amount of normal plasma is mixed with the plasma of vitamin K-deficient chicks the clotting time is accelerated, depending upon the amount of prothrombin added. On standing several hours before the addition of tissue factor no change in activity occurs in these plasma mixtures. This experiment indicates that the addition of active plasma does not cause an activation of an inactive prothrombin in the plasma of the vitamin K deficient birds. There is, therefore, no evidence indicating that prothrombin is present in the blood of vitamin K-deficient chicks in an inactive form.

Determination of prothrombin. a. Determination of activity of tissue factor. The prothrombin concentration of deficient plasma may be expressed in terms of the percentage of prothrombin which is normally found in the plasma of healthy chicks. By mixing the plasma of normal animals with varying amounts of the plasma from vitamin K-deficient birds, it is possible to demonstrate that the clotting time is dependent upon the concentration of prothrombin, provided the concentration of added tissue

factor remains constant. One unit of tissue factor may be defined as that amount of the agent necessary to obtain a clotting time of 180 seconds in normal plasma.

The following experiment illustrates the method used to standardize a typical tissue extract. Several dilutions of tissue extract in Ringer's solution were prepared and one drop of each dilution added to 0.2 ml. of normal chick-plasma and the clotting time recorded.

CONCENTRATION OF TISSUE FACTOR	CLOTTING TIME
per cent	*seconds*
0.25	360
0.50	240
1.00	165
2.50	115

The amount of clotting agent capable of clotting the plasma in 180 seconds is now readily determined by plotting the log concentration of tissue factor against the log of the reciprocal of the clotting time. These functions are derived from Fischer's equation $\frac{1}{t} = KC^a$ (6), which attempts to relate clotting time, t, with the concentration of the tissue factor, C. In the above equation, K and a are constants. It was found in the above experiments that one drop of a 0.91 per cent solution of tissue factor contains one unit of clotting agent. The prothrombin levels of the plasma of normal chicks may differ by as much as 100 per cent. Therefore in general three normal animals have been used in order to determine the strength of the clotting agent. Only fresh plasma is used. However, the differences between normal animals are small and especially when compared to the differences between normal and K-deficient animals. By introducing more clotting agent into the plasma with a low prothrombin content it is possible to shorten the clotting time to 180 seconds.

b. Relationship between tissue factor and prothrombin. The following experiments show the relation between the prothrombin content of different plasmas and the amount of tissue factor necessary to clot plasma in 180 seconds. Varying quantities of normal plasma were mixed with that of animals suffering from a pronounced vitamin K-deficiency. The number of units of tissue factor necessary to cause clotting in 180 seconds were then determined. The plasma of such birds contains but small traces of prothrombin, and the amount is negligible in comparison with the amount of added prothrombin from normal plasma.

The experiments recorded in table 2 show that the percentage of prothrombin, when multiplied by the units of tissue factor necessary to cause clotting in 180 seconds, is constant or = 100. It is possible therefore to

calculate the amount of prothrombin in any given plasma by determining the units of clotting agent necessary to clot the plasma in 180 seconds. This principle has been used in the following experiments.

The lack of identity of prothrombin and complement. Bordet (7) as well as Hirschfeld and Klinger (8) have recognized a possible similarity between complement and prothrombin. Fucks and van Falkenhausen (9) have attempted to prove that prothrombin is identical with the mid-piece of complement, although Eagle (10) has questioned the validity of their

TABLE 1

Clotting time of normal and vitamin K deficient chick plasma

PLASMA	TIME AFTER MIXING	CLOTTING TIME	
	minutes	*seconds*	
a	2	165	165
a	60	165	165
a	90	165	180
a	120	165	180
b	2	1,800	1,860
b	60	1,710	1,860
b	120	1,800	1,920
c	2	870	780
c	16	810	840
c	35	810	840
c	50	840	900
c	110	840	780
c	130	840	900

a = Normal plasma mixed with equal volume of Ringer's solution.

b = K-avitaminous plasma mixed with equal volume of Ringer's solution.

c = K-avitaminous plasma mixed with equal volume of Ringer's solution containing 2 per cent normal plasma.

TABLE 2

Units of tissue factor necessary to produce clotting with varying concentrations of prothrombin in 180 seconds

PROTHROMBIN IN PER CENT OF NORMAL	UNITS OF TISSUE FACTOR NECESSARY TO CAUSE CLOTTING IN 180 SECONDS
100	1.00
50	1.75
33	3.3
25	3.8
16.7	6.6
12.5	7.8
8.4	12.3
6.25	20.0
5.0	20.0
4.2	22.0
2.0	66.0
1.0	100.0
0.5	170.0
0.25	400.0
0	800

experimental results. More recently Bancroft, Quick and Stanley-Brown (11), as well as Quick (12), have reopened this question and have concluded that prothrombin and complement are not identical. Their conclusion is based upon the fact that aluminum hydroxide will remove or inactivate prothrombin, but does not affect the titer of complement. The following experiments were carried out to investigate this possible relationship.

Determination of complement. Normal chicken plasma does not contain complement capable of acting in a system consisting of sheep cell and

sheep-cell amboceptor. It is, however, known that chicken blood contains a hemolysin against rabbit cells (13). It is possible to separate this hemolysin into an amboceptor and complement part by the technique of Ehrlich and Morgenroth (14). The following system was used to determine the hemolysin concentration in chicken plasma. To 0.3 cc. of a 2 per cent suspension of rabbit cells was added 0.3 cc. of plasma in varying dilutions. In all experiments the red cells were taken from the same rabbit. The least amount of chicken plasma expressed in volume per cent necessary to give complete hemolysin in half an hour was determined. All experiments were conducted at 37°C.

TABLE 3

Prothrombin and hemolysin concentration in normal chicken plasma

CHICK NUMBER	AGE	WEIGHT	PLASMA PROTHROMBIN IN PER CENT OF NORMAL	LEAST AMOUNT OF PLASMA GIVING COMPLETE HEMOLYSIS
	days	*grams*		*volume per cent*
1	3	44	100	
2	3	44	100	
3	5	48	100	
4	6	52	100	33
5	8	60	100	33
6	12	64	100	33
7	12	68	100	16.5
8	16	100	80	12.5
9	20	140	80	8.3
10	24	145	100	12.5
11	24	155	100	8.3
12	28	120	100	6.3
13	30	200	90	12.5
14	30	310	100	12.5
15	?	600	80	4.2

It is to be expected that large differences in the complement concentration would be reflected in the hemolytic activity of the plasma.

Tables 3 and 4 show that even though the concentration of prothrombin in the different plasmas varies, there does not seem to be any pronounced variation in their hemolytic activity. In K-deficient chicks with low prothrombin content the hemolytic activity is normal, and in a young animal with low hemolysin concentration (whether due to a low complement or amboceptor concentration or both) the prothrombin concentration is likewise normal. The conclusion may therefore be drawn that there is no identity between complement, or parts of complement, and prothrombin.

The species specificity of avian prothrombin. No experimental evidence

may be found in the literature concerning the species specificity of prothrombin in chickens. In order to determine the prothrombin specificity of various species the following experiments were performed.

Vitamin K-deficient chicks were transfused with the blood plasma from various normal animals and the amount of prothrombin determined before and after transfusion. The 0.3 per cent citrated blood from human, dog, rabbit, pigeon was removed by venous puncture, centrifuged and freed

TABLE 4

Prothrombin and hemolysin concentration in plasma of vitamin K-deficient chicks

CHICK NUMBER	AGE	WEIGHT	UNITS OF AGENT NECESSARY TO CLOT IN 180 SECONDS	PLASMA PROTHROMBIN IN PER CENT OF NORMAL	LEAST AMOUNT OF PLASMA GIVING COMPLETE HEMOLYSIS
	days	*grams*			*volumes per cent*
16	20	96	10	10	16.5
17	48	200	5	20	6.3
18	48	200	145	0.7	12.5
19	30	128	15	6.7	12.5
20	44	208	46	2.2	6.3
21	24	138	65	1.5	8.3
22	40	160	50	2.0	12.5
22*	43	160	1	100.0	12.5
23	27	140	270	0.37	12.5
23†	30	148	1	100	4.2
24	48	180	70	1.4	8.3
24‡	51	185	1	100.0	12.5
25	32	160	100	1.0	12.5
25§	35	160	1.5	67	12.5

* After 3 days' administration of vitamin K, number 22 received dried spinach, in all 500 vitamin K units.

† After 3 days' administration of vitamin K, number 23 received dried spinach, in all 1000 vitamin K units.

‡ After 3 days' administration of vitamin K, number 24 received spinach extract, in all 10,000 vitamin K units.

§ After 3 days' administration of vitamin K, number 25 received spinach extract, in all 200 vitamin K units.

from all cellular elements by passing through a Berkefeld filter. Blood from the duck and the chicken was removed by bleeding from the carotid, that from eels by cutting the tail, and that from the frog by decapitation. The plasma was infused slowly through the wing vein. The amount given was 1 per cent of the body weight. Clotting determinations were made two hours after the transfusion. All experiments were conducted in duplicate.

On introducing plasma from the eel and frog into K-deficient chickens it was found that the animals died immediately. Three animals were injected with a small amount of each of these plasmas (in one case only 0.1 cc. of eel plasma being used) yet in every instance the animals died within about ten minutes. Death seemed to occur sooner with eel plasma than with that of frog. When normal animals were used the same toxic effect was observed. The death seems not to be due to intravascular clotting.

From table 5 it may be seen that the plasma of the chicken, duck and pigeon contains prothrombin capable of substituting for the prothrombin lacking in the vitamin K-deficient animals, whereas that derived either from the human, rabbit or dog is not.

It is also of interest to observe whether chicken prothrombin may act in the clotting process of human, dog and pig plasma. This question

TABLE 5

Prothrombin content of vitamin K-deficient chicks after transfusion with plasma of various species

CHICK NUMBER	WEIGHT	PROTHROMBIN BEFORE TRANSFUSION	TRANSFUSED WITH	PROTHROMBIN AFTER TRANSFUSION
	grams	*per cent*		*per cent*
1	260	2	2.6 cc. chicken plasma	50
2	400	1	4.0 cc. duck plasma	50
3	380	1	3.8 cc. pigeon plasma	50
4	500	1	5.0 cc. rabbit plasma	1
5	500	1	5.0 cc. dog plasma	1
6	570	1	5.7 cc. human plasma	1
7*	270	100	2.4 cc. 0.3 per cent citrate solution	100

* Control animal showed no effect from the administration of citrate alone.

was investigated in the following way. The prothrombin from oxalated human, dog and pig plasma was absorbed with aluminum hydroxide according to the method Quick (15). Only a part of the prothrombin could be removed. The oxalated plasma contains the clotting agent itself in the form of blood-platelets, and will spontaneously clot by the addition of an appropriate amount of $CaCl_2$. The results of those experiments appear in table 6. The prothrombin in this experiment was prepared by Howell's acetone method (16).

The results of the experiments given in table 6 are supplementary to those presented in table 5 and indicate that avian prothrombin derived from chicken and pigeon plasma is inactive in a system consisting of the fibrinogen and homologous tissue factor of human and dog plasma. Similar results were obtained by using absorbed pig plasma. There seems to

be no difficulty, on the other hand, for mammalian prothrombin to act in systems of human, dog and pig "aluminum"-plasma.

Discussion. The first experiments in this study indicate that there is no evidence pointing to the fact that prothrombin is present in the plasma of vitamin K-deficient chicks in an inactive form. It is obvious, therefore, that hemorrhagic diathesis is caused by a decrease in plasma prothrombin. The clotting anomaly in vitamin K-deficiency is apparently less complicated than is that in *hemophilia congenita*, as it has been found that all other factors involved in the clotting mechanism are normal (1.2). It has also been possible to demonstrate that there is no increase in the

TABLE 6

Species specificity of prothrombin of various species

ADSORBED PLASMA* (0.5 cc.)	SOURCE OF PROTHROMBIN (0.2 cc.)	CLOTTING TIME†	
species	*species*	*minutes*	
Human	Human	6	6½
	Cat	5	5
	Dog	5	5½
	Pig	2¼	2¼
	Chicken	28	30
	Pigeon	32	35
	0.9 per cent NaCl	32	32
Dog	Human	3½	3
	Cat	2¼	2¼
	Dog	2¼	2¼
	Pig	1	1
	Chicken	8	9
	Pigeon	8	9
	0.9 per cent NaCl	8	9

* i.e., plasma from which major portion of prothrombin has been adsorbed.

† After addition of 0.3 cc. of 0.5 per cent $CaCl_2$ solution.

anticlotting power of the plasma of vitamin K-deficient chicks (1, 2). The salts and plasma proteins are apparently present in normal concentrations (17).

Differences in the case of activation of prothrombin play an important rôle in the clotting theory of Bordet (18), who finds that the prothrombin present in serum is more readily activated by the tissue factor and calcium ions than is the prothrombin in plasma. He suggests the name *proserozym* for the factor in plasma and *serozym* for the prothrombin in serum.

A simple quantitative measurement of the prothrombin concentration in normal and vitamin K-deficient birds has been established. If the average prothrombin content of normal animals is considered as 100 per

cent, the relationship between the prothrombin concentration and units of clotting agent necessary to clot the plasma in 180 seconds seems to be constant. The average prothrombin content of normal plasma must be used as a provisional standard until stable prothrombin or tissue factor preparations become available. In vitamin K-deficiency there is a decrease in the prothrombin concentration which varies in pronounced cases from nearly 0 to 2 per cent of normal.

It is of interest that Brinkhous, Smith and Warner (19) have recently reported that the level of prothrombin in newborn babies is between 14 to 39 per cent of the level found in the plasma of normal adults. This phenomenon may possibly have some connection with vitamin K-deficiency. The prothrombin content in healthy 3 to 4 days old chicks was found to be normal.

It has been shown that the prothrombin and complement of chicks are not identical. The hemolytic activity of normal plasma is independent of variations of prothrombin concentrations. Some investigators have noted a low complement concentration in guinea pigs suffering from deficiency of vitamin C (20, 21, 22) and in vitamin A deficiency (23). This phenomenon does not seem to occur in vitamin K deficiency.

From the experiments recorded in tables 5 and 6 it is apparent that a distinct species specificity is exhibited by the prothrombin of chick-plasma. It appears that the prothrombin of those birds for which vitamin K is directly necessary (24) can act in the clotting process of chick blood. On the other hand prothrombin from mammals (for which the K-factor does not seem to be necessary) cannot act in the clotting of chicken plasma. In spite of the species specificity of the prothrombin of birds Greaves and Schmidt (25) have evidence that vitamin K takes part in the formation of prothrombin in rats.

SUMMARY

1. The hemorrhagic diathesis in chickens produced by a deficiency of vitamin K is due to a decrease of plasma prothrombin. In such animals prothrombin is not present in an inactive form.

2. A quantitative method for the determination of the prothrombin content in chicken plasma is outlined. A direct relationship exists between clotting time, concentration of prothrombin and tissue factor. In producing the same clotting time, the product of the clotting agent and the concentration of prothrombin is constant.

3. The prothrombin content of the plasma of very young chicks is the same as that found in adult birds.

4. Prothrombin of chicks is not identical with the mid-piece of complement. Vitamin K-deficiency does not affect the concentration of complement.

5. Chicken prothrombin appears to be a species specific substance for birds.

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THE EFFECT OF THE REMOVAL OF THE SUPERIOR CERVICAL GANGLION ON LACHRYMAL SECRETION

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Little is known concerning the effect of degenerative section of the sympathetic nerve supply to glands. Although Sharpey-Shafer (1924) states that the salivary glands are especially sensitive to adrenalin after denervation, no conclusive evidence has yet been advanced as to the correctness of this statement. A survey of the question related to observations on salivary secretion after denervation will be given elsewhere (Simeone and Maes, 1938). The present paper deals only with the secretory response of the lachrymal gland to drugs before and after removal of the superior cervical ganglion.

METHODS. Cats were used under nembutal anesthesia (0.5 to 0.7 cc. of a 6 per cent solution, per kgm. intraperitoneally). To detect the lachrymal secretion use was made of dry strips of white, smooth blotting paper $\frac{3}{4}$ inch long and $\frac{1}{2}$ inch wide. At the start of an experiment a strip was placed under the upper lid of each eye and removed 4 minutes later. Thus any residual secretion would be absorbed and the subsequent readings would be made in standard conditions. Ten minutes later two new strips, which had been weighed in glass-stoppered weighing bottles, were likewise inserted under the upper eyelids and left in place for 4 minutes. Ten minutes later the process was repeated, and so on.

Usually two or three readings were thus taken before the injections of adrenalin, pilocarpine or acetylcholine. These were then made in the femoral vein one minute before the blotting paper was inserted in the eye.

Two series of animals were studied. The first consisted of four cats. In these animals the responses to adrenalin, pilocarpine and acetylcholine were recorded, then one superior cervical ganglion was removed. Fifteen to thirty minutes later the procedure of injections and recordings was repeated in every point identical to the first.

The second series consisted of six cats. Three of these had normal adrenals. In the other three one adrenal had been demedullated one month previously and the other removed two weeks before the start of the experiment. Demedullation was carried out according to a technique

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generally used in this laboratory. The gland was dissected free from the surrounding connective tissue, slit open in a frontal plane, the medulla scraped out with a curette and removed by low pressure suction. The opening in the gland was stitched. These six cats were studied in the same way. The response to drugs was measured two or three times at several days' interval. Then under ether anesthesia the left superior cervical ganglion was removed aseptically. The cats were let recover fully from the operation. Observations were only taken when no hyperemia of the conjunctiva and no inflammation of the eyelids were visible. For these reasons the experiment was usually discontinued for a period of 11 days following the operation. At different intervals the responses of the two glands to injected drugs were again compared in the same way as described above.

Results. The amount of secretion present under the eyelids when no drugs had been injected varied from 6 to 20 mgm. In all animals intravenous injections of 0.1 mgm. pilocarpine per kgm. body weight caused a marked increase in secretion, often 3- to 6-fold the original value. Adrenalin and acetylcholine as a rule gave a definite increase only when rather strong doses were administered, i.e., 0.5 mgm. acetylcholine per kgm., injected intravenously during a period of 15 seconds, and 50 to 75 γ adrenalin per kgm. body weight, administered in the same way.

In the four animals in which the immediate effect of the removal of the superior cervical ganglion was studied, no difference in response between the two eyes could be detected either before or after the ganglionectomy (see fig. 1). The acutely denervated gland, therefore, does not appear to be sensitized to adrenalin, pilocarpine or acetylcholine.

In the second series it was found that in three animals the secretion before the operation was identical (within the limits of experimental error) on both sides. In the three other cats the right gland secreted more, regardless of the presence or the absence of the adrenal medulla. Nevertheless, when tests were made at different intervals during the period from 11 days to 8 weeks after removal of the left superior cervical ganglion, the denervated gland invariably showed a marked increase in responsiveness to the injected drugs: 1, it presented a definite response to a dose which was subliminal for the normal side; and 2, its response to a dose effective on both sides was constantly larger than the normal (see figs. 2 and 3).

This sensitization as it appears from the charts is striking but cannot be estimated quantitatively. For it should be emphasized that the size of the responses to a given dose of the same drug differs not only from one animal to another, but in one cat varies from one experiment to another. The reasons for this variability are not clear, although the depth of the anesthesia appears to be an important factor in regulating the intensity

of the response; light anesthesia has been noted as favoring free secretion from a gland which, under deep anesthesia, produced hardly enough fluid to moisten the eye.

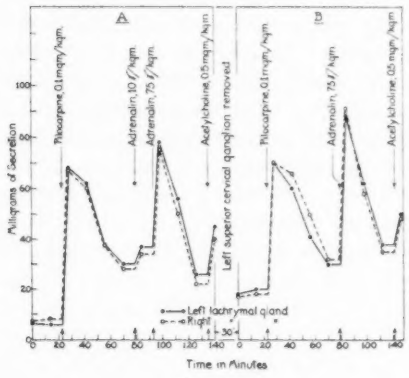

Fig. 1

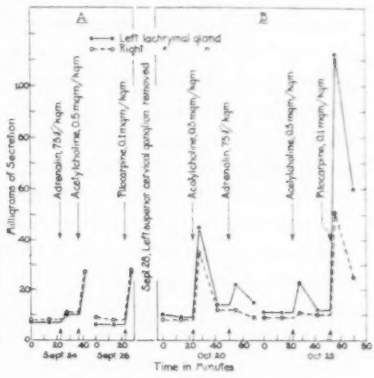

Fig. 2

Fig. 1. Experiment, November 12. Normal cat, adrenals intact. Curves showing responses of both lachrymal glands to different drugs before and immediately after removal of left superior cervical ganglion. It is assumed (as in figs. 2 and 3) that between the moment of the injection, as indicated by the arrow, and the moment when the last reading was made the secretion has not changed.

Fig. 2. Cat. Left adrenal out, right adrenal demedullated. Successive responses of both lachrymal glands to injected drugs at indicated dates. *A*, before, and *B*, after, removal of left superior cervical ganglion.

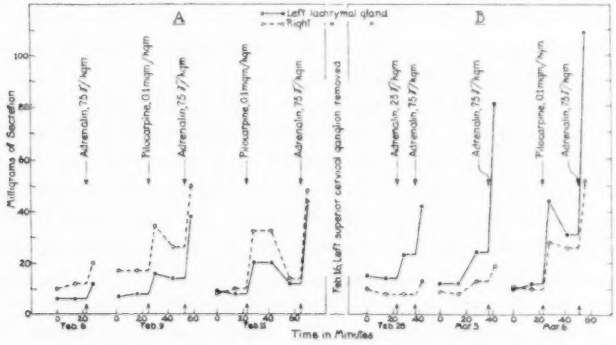

Fig. 3. Cat. Adrenals intact. Same as figure 2

Discussion. Langley (1901) studying the effect of adrenalin on different organs in the cat reported one experiment in which the secretion of tears elicited in both eyes by this drug was markedly greater on the side

where the superior cervical ganglion had been removed eight days previously. Our results appear to confirm that single observation.

But between the work of some of the older authors and ours there are discrepancies which may be now considered. In our acute experiments we did not find any immediate changes in the lachrymal secretion after removal of the superior cervical ganglion. On the other hand Arloing (1890) and Levinsohn (1903) have reported that a few minutes after the resection of the cervical sympathetic the corresponding eye is filled with tears. Schirmer (1909), who could not confirm these results, suggested that the observed secretion was produced by reflex stimulation, due to hyperemia of the conjunctiva. If this is true it is obvious that we did not observe any change in secretion because the acute experiments were carried out under deep anesthesia with nembutal, which suppressed all reflex secretion of tears. On the other hand, ether is known almost always to cause secretion of tears in the eyes of normal cats. In our aseptic operations, therefore, it would have been impossible to find grossly any difference between the two eyes. In a few cases, however, in which for some days after the operation tears were present in the denervated eye, conjunctivitis was invariably present.

Sensitization of the lachrymal gland to pilocarpine 24 hours after removal of the superior cervical ganglion was observed by Arloing (1890) in the unanesthetized horse. On the contrary, Merz (1926) the fifth day after denervation could not detect in the anesthetized rabbit a similar sensitization. In one experiment in which we made a test 4 days after removal of the superior cervical ganglion we did not get evidence of increased responsiveness to pilocarpine or adrenalin. Arloing's results might have been due to conjunctivitis. But it is possible also that he detected the early stage of sensitization which had not become apparent in Merz' experiments nor in the one just reported.

The phenomenon with which we are dealing resembles closely the sensitization of smooth muscle to adrenalin described originally by Anderson (1903) and by Meltzer and Auer (1904) (for recent review see Hampel (1935) and Simeone (1936)). Both are absent immediately after denervation, and become evident after a few days. But why, after sympathetic denervation, do the glands become sensitive to parasympathetic drugs? The idea might be favored that the sensitization to pilocarpine and acetylcholine is not direct but due to liberation of adrenine from the adrenal medulla, which those drugs stimulate. That this supposition is incorrect is proved by the fact that in cats with one adrenal out and the other demedullated the phenomenon appears exactly as in cats with normal adrenals. It should be remembered, too, that this multiple sensitization is not unique. Rosenblueth (1932) observed it likewise for the smooth muscle of the nictitating membrane, in which he found, after degenerative

section of the cervical sympathetic, increased responsiveness not only to adrenalin but to acetylcholine, pilocarpine, physostigmine and histamine.

The explanation of the phenomenon is not clear. It is probably analogous to the sensitization of the submaxillary gland to pilocarpine recently reported by Pierce and Gregersen (1937) after section of the chorda tympani. But in the absence of enough data on the subject we shall not attempt to offer an explanation. Until new findings change our experimental outlook, the theory presented by Rosenblueth and Cannon (1936), explaining sensitization of smooth muscle after denervation by an increase in the permeability of the cells may be considered as a convenient working hypothesis.

SUMMARY

The secretion of the lachrymal gland in response to injections of acetylcholine, pilocarpine and adrenalin has been studied in cats.

Removal of the superior cervical ganglion causes no immediate change in the response of the denervated gland to those drugs (see fig. 1) but if tests are made 11 days or more after the operation an increase in sensitivity can be observed (see figs. 2 and 3).

Some differences and some correlations between our findings and those reported by older authors are discussed. The analogy is presented between the phenomenon described here, the well-known sensitization of smooth muscle to drugs after degenerative section of the sympathetic and the increased sensitivity of the submaxillary gland to pilocarpine after section of the chorda tympani (cf. Pierce and Gregersen, 1937).

The theory that these changes are due to increased permeability of the cells, proposed by Rosenblueth and Cannon (1936) is adopted as a working hypothesis.

It is a pleasure to thank Dr. W. B. Cannon for the suggestion of this problem and for the active interest he took in its investigation.

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THE RECOVERY OF AN ADRENALIN-LIKE SUBSTANCE FROM THE KIDNEY[1]

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Hypertension may be produced in animals by certain procedures which interfere with the renal blood flow (1) (2) or with the escape of urine (3) (4). The rise in blood pressure so caused appears to be due—either directly or indirectly—to substances formed in the kidney, for it persists after renal denervation (2) (5), after complete resection of the sympathetic nervous system (6) and even after transplantation of the kidneys (5); but disappears when the affected kidneys are removed (5). It has been shown that kidney tissue contains a pressor substance (7) and that the amount of this substance seems to be increased in certain types of renal hypertension (8) (9) (10). However, attempts to demonstrate a pressor action from blood obtained from the renal veins of animals with experimental renal hypertension have yielded negative results (11). Since the blood flow through the kidneys is very large in proportion to their size (12) (13) it seems possible that the failure to find pressor effects from renal vein blood may be due to dilution of a small amount of pressor substance with a large amount of blood. Such a difficulty might be overcome by perfusing kidneys removed from the body, using a small volume of perfusion fluid relative to the size of the kidneys. The purpose of this communication is to report a study of the pressor properties of material obtained in this way.

Kidneys of hogs were obtained from the slaughter house, care being taken to avoid injury to the renal artery and capsule. A cannula was tied into the artery and Ringer's solution was slowly perfused from a syringe through the kidney, the outflow from the renal vein being collected by means of a funnel.

The pressor property of the perfusate was determined on rats anesthetized with sodium pentobarbital. Blood pressure was measured with a small bore mercury manometer attached by means of a three-way tap to a needle inserted into the lower portion of the abdominal aorta. Injections of one-half to one cubic centimeter of the renal perfusate were made into the side connection of the tap. The slight rises in blood pressure caused by the volume of fluid injected were determined by injecting equal amounts of 0.9 per cent sodium chloride solution.

[1] This work was aided by a grant from the Josiah Macy, Jr. Foundation.

In order to find out whether the effects observed from renal perfusate were specific, similar measurements of blood pressure were made following the administration of material obtained by perfusing lung, liver and spleen.

The results of the injection of the renal perfusate were striking. An immediate rise in blood pressure occurred which reached its peak within fifteen seconds after the end of the injection. The perfusate of other organs had no greater pressor effect than did an equivalent volume of salt solution (fig. 1).

Re-perfusion through the same kidney of the first perfusate obtained had only a slight effect in increasing the potency. After repeated per-

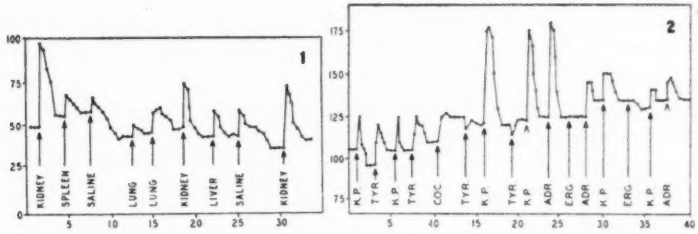

Fig. 1. Comparison of pressor effects of saline and of perfusates of spleen, lung, liver and kidney. Perfusates of spleen, lung and liver caused no greater rise in blood pressure than did equal quantities of saline solution. Definite pressor effects characterized by sharp evanescent rise in blood pressure were obtained from renal perfusate. Minutes are plotted on the abscissa, mean arterial blood pressure on the ordinate.

Fig. 2. The effect of cocaine and ergotamine on the renal perfusate as compared with tyramine and adrenalin. *K.P.* = 0.5 cc. kidney perfusate; *Tyr.* = 0.1 mgm. tyramine; *Adr.* = 0.0004 mgm. adrenalin; *Coc.* = 5 mgm. cocaine; *Erg.* = 0.05 mgm. ergotamine. The pressor response to tyramine is seen to be slower in onset and more prolonged than that of renal perfusate. (The latter material had been heated in order to destroy the renin.) Cocainization abolished the pressor effect of tyramine but enhanced the action of renal perfusate. The blood pressure responses to adrenalin and to renal perfusate were similar and were decreased to about the same degree by ergotamine. Minutes are plotted on the abscissa, arterial blood pressure on the ordinate.

fusion of a given kidney with fresh Ringer's solution the perfusate became inactive.

Since the kidneys of hogs could not be obtained from the slaughter house and perfused until an hour or more after the death of the animal, freshly removed kidneys of dogs were tested. When the perfusion was carried out within a few minutes after the removal of the organ, smaller pressor responses were obtained than after the same kidneys had stood in the room (at about 25°C) for two hours or more. The rise in blood pressure usually obtained was of the same general magnitude as that pro-

duced by equal volumes of one to one million to one to ten million adrenalin solutions.

In some instances the renal perfusate produced only evanescent rises in blood pressure which returned to the previous level within one to three minutes. More frequently, however, the initial sharp increase in blood pressure was followed by a slight rise which persisted for five to fifteen minutes. Further observations indicated clearly that the effects were due to two pressor agents, the immediate sharp and evanescent effect being dependent on a heat-stable, dialysable substance, while the less marked but more sustained pressor action was due to a heat-labile, non-dialysable compound (fig. 3). The former substance was found to be soluble in 85 per cent alcohol; the latter was insoluble in such a solution but could be recovered from the precipitate. Judging from its pro-

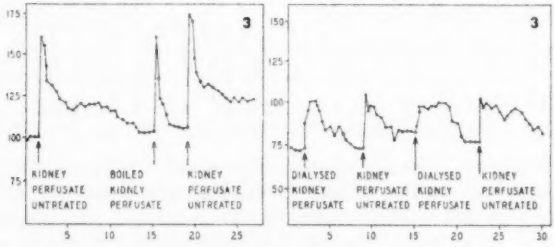

Fig. 3. The effect of boiling and of dialysis on renal perfusate. A. Left. Boiling renal perfusate had practically no effect on this immediate pressor response but did abolish the prolonged response produced by the untreated extract. B. Right. Dialysis, on the other hand, abolished the immediate effect but did not diminish the delayed sustained effect. Minutes are plotted on the abscissa, arterial blood pressure on the ordinate.

longed but relatively slight pressor effect, this alcohol-insoluble, heat-labile, non-dialysable compound appears to be identical with the pressor substance (Tigerstedt's "renin"), which can be obtained by maceration of renal tissue. When saline extracts of such tissue are made the predominant pressor effect is due to renin, but one sometimes observes marked initial rises which are similar to those obtained from renal perfusate. In the latter material the predominant effect is the quick response but slight persistent renin-like effects are usually observed also. Since the substance which causes the evanescent pressor response is readily dialysable it is not surprising that this effect predominates in the material obtained by perfusing the kidney.

The observations mentioned in the preceding paragraph indicate, we believe, that under appropriate conditions two different blood pressure raising agents can be obtained by perfusing the isolated kidney. One of

them seems, from the available evidence, to be identical with the substance described by Tigerstedt and Bergmann (7) and named by them "renin." In order to obtain information concerning the nature of the other (quick-acting pressor agent) further experiments were done. Comparisons were made of the character of the pressure curve following the injection of *a*, heated perfusate; *b*, tyramine monohydrochloride, and *c*, adrenalin hydrochloride. It was found that tyramine caused a less rapid and more sustained rise in blood pressure. The response to the heated renal perfusate was, however, quite similar to that of adrenalin (fig. 2). Furthermore, doses of cocaine which abolished the pressor response to tyramine enhanced the effect of the other substances. Finally, ergotamine tartrate caused marked and comparable diminution in the pressor response to adrenalin and to the heated renal perfusate (fig. 2).

Observations were also made on the isolated leg of the rat, vasoconstriction being determined by measurement of the rate of entry of fluid into the cannulated femoral artery. Pronounced vaso-constrictor effects were obtained from the heated renal perfusate, indicating that the pressor effects of this material are due to local action on the vessels rather than to stimulation of the nervous system.

These observations indicate, we believe, that—insofar as can be judged from its pressor effect on the rat—the heat-stable substance obtained by perfusion of the kidney is either adrenalin, sympathin, or some unknown adrenalin-like compound. Until more definite knowledge of the chemical structure of this substance becomes available we have chosen to call it "perfusin."

Perfusin solutions were found to cause marked dilatation of the pupil of the enucleated frog's eye, the effect being comparable to that of 1:10-000,000 solution of adrenalin hydrochloride.

We were interested in finding out whether any relationship exists between perfusin and renin, i.e., whether the former is formed by the decomposition of the latter. Experiments designed to test this point did not yield conclusive results.

The evidence quoted indicates that under certain conditions pressor substances may be liberated from the kidney tissue into the renal vessels. Whether or not such liberation is in any way concerned with the pathogenesis of renal hypertension is not known at the present time.

SUMMARY

Two pressor substances may be obtained by perfusion of isolated kidneys of hogs and dogs. These are not present—in detectable amounts—in the perfusates of spleen, liver and lung. One of these pressor substances is heat-labile, alcohol-insoluble, non-dialysable and produces a prolonged but slight rise in blood pressure. This substance appears to be similar

to Tigerstedt's renin and is less concentrated in renal perfusate than in extracts of macerated renal tissue.

The other substance, which can be obtained in greatest concentration by perfusing a kidney which has been removed from the body an hour or longer, resists boiling, dialyses readily, and is soluble in alcohol. Its pressor effect is enhanced by cocaine and diminished by ergotamine. The blood pressure curve following its administration is similar to that following the injection of adrenalin. It is believed that this substance (which for convenience is called "perfusin") is either adrenalin, sympathin or some other adrenalin-like substance.

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OBSERVATIONS ON WATER METABOLISM IN THE DESERT

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It is well known that water becomes an exceedingly important item of existence in dry, hot climates. Everyone who goes to the desert becomes water-conscious; drinking habits are modified, and various other features of water exchange which are less frequently in consciousness are changed.

The present data were obtained by the Harvard Desert Expedition in the summer of 1937 at Boulder City, Nevada. The seven members of the expedition were the subjects,[1] since it was desired to follow changes as they might develop during the stay in the hot climate. With this in mind, certain previous and subsequent observations were made on each of the subjects (mostly in Boston); these furnish bases of comparison.

Each of the subjects followed approximately the same routine. The mornings were usually spent in laboratory activities. The afternoons were spent in exercise tests lasting one or two hours, or in other tests which took their place; each member of the party also making and recording measurements on others. Approximately a uniform amount of outdoor exercise was undertaken, each subject choosing once for all a duration and pace for it. Various portions of the plan included studies of heat balances, chloride balances, blood changes, and effects of exercise. These studies will be separately reported; the present paper is concerned only with water balances.

The conditions of climate were such as to allow maximal rates of evaporation without the sweat appearing upon the skin. The exposures during which perspiration was measured were ordinarily at the hottest part of the day and in direct sunlight. The mean shade temperature for these exposure periods was 35.3°C. (96°F.) with a maximum of 43°C. The mean humidity was 12 per cent and it varied from 6 to 30 per cent. Radiation was usually undisturbed by clouds. Winds were relatively rapid,

[1] The authors are grateful to the other members of the Expedition for their careful collaboration. They were: F. G. Hall, H. T. Edwards, R. A. McFarland, F. Consolazio, and D. B. Dill, Jr. Space for a laboratory, and other facilities, were provided through the coöperation of Mr. John Page, Commissioner, Mr. Ralph Lowry, Resident Engineer, and Mr. Ely, Boulder City Manager, of the U. S. Bureau of Reclamation.

the mean velocity being 137 meters per minute throughout the twenty-four hours.

Since the object of the studies of water metabolism was to find what changes occurred as the subjects became accustomed to life in the hot dry climate, most of the subjects were not exposed to hot conditions until they arrived in Boulder City. Several of the adjustments that could be traced in the measurements appeared to take place immediately. These adjustments were those of increased evaporation, decreased urine output, and increased intake of water.

Several slower adjustments were brought to light. In two subjects marked increases in rates of sweating in response to the same thermal and exercise stimulus gradually developed (Adolph, 1938). Second, the dilution of the sweat increased over a period of from 1 to 10 days in diverse subjects (Dill, Hall and Edwards, 1938). Third, the rate of ingestion of water, just after a considerable loss of it, increased.

TABLE 1

Mean rates of water output for each subject

	A	C	D	E	H	J	M	MEAN
Days observed	31	32	23	24	31	4	11	22
Total day's urine, cc. per hour	48	37	26	38	37	29	27	34
Its specific gravity	1.025	1.031	1.030	1.025	1.025	1.031	1.032	1.029
Urine in exercise, cc. per hour	46	40	20	45	40	13	33	32
Its specific gravity	1.029	1.034	1.033	1.030	1.030	1.032	1.033	1.032
Evaporation in exercise, cc. per hour	970	1010	1070	980	790	1250	1130	1035

Water output. Outputs in urine and in evaporation were compared *a*, in the brief periods of one or two hours during which muscular exercise was performed outdoors, and *b*, during the 24 hours, much of which was spent indoors in comparatively cool environments (table 1). Higher rates of urine output tended to occur on days of low water ingestion, hence of low rates of sweating (less exercise and exposure). Rates of urine output did not vary much with daily or with hourly temperatures. No significant differences appeared in the rates of urine formation in exercise compared to the rates for the rest of the day. Mean evaporation rates, on the other hand, were 4 or 5 times more rapid during exposure than during other hours. On most days evaporation during exercise was some 30 times the rate of urine formation; in individual observations it was 60 times as rapid.

For each of the subjects, rates of evaporation may be compared for varying manners of exposure (table 2). Temperature of air (Adolph, 1938), radiation, and rate of exercise are the factors which could be differ-

entiated. Even indoor activities required considerably greater expenditures of water than the same activities in other climates. The mean temperature in the laboratory was 30°C. and the mean relative humidity was 35 per cent.

The relation of rate of evaporation during an hour's exposure to daily temperature of air is that which might be anticipated (Adolph, 1938). However, not all of the subjects showed the greatest 24-hour expenditure of water upon the hottest day. This was partly due to the facts that muscular activities varied, and that indoor conditions did not always run parallel to those outdoors.

TABLE 2

Rates of evaporation under various conditions, in grams per hour, for each subject

	A	C	D	E	H	M	MEAN
Indoors	200	200	260	210	230		210
			140				
Sit in shade		450	400		450	530	430
		330			430		
Sit in sun	710	730	630	720	570	820	680
	590	690	720		600	690	
Slow walk in sun	750	820	930		570		800
		910	920		700		
Walk in shade	870	940	900	860		840	870
	910	770		810			
Walk in sun	1010	1280	1070	960	1040	1240	1120
	1210	1220	1270	950	1050		
Maximum measured	1440	1470	1700	1290	1430	1650	1500

The maximum rates of evaporation were, in diverse subjects, 1300 to 1700 grams per hour (table 2). These are similar to the maximal rates of sweating which have been reported by other authors (Kuno, 1934).

At Boulder City daily measurements were made (by the hydrographer for the Boulder Dam project) of rates of evaporation of water from a large pan exposed to sunlight (hook-gauge method). The maximum rate of evaporation for any 24 hour-period was 0.090 cm. per hour. In one hour's exposure subject D evaporated a maximum of 1700 cm.^{3} per 19,600 cm.^{2} of body surface, or 0.087 cm. per hour. The identity of rates is mainly fortuitous; it indicates that the human surface loses less water than the fully radiated water surface at the hottest part of the day.

The losses of dissolved materials in sweat and in urine are often reciprocal to one another (Dill, Hall, and Edwards, 1938). The concentration of chloride in the sweat was always considerably smaller than the concentration of chloride in the blood serum. In urine the concentration of chloride was maintained above that of serum except upon a few days in which one subject in particular had almost chloride-free urine.

The concentration of the urine as indicated by its specific gravity (table 1) was significantly greater during exercise than during the rest of the day, in spite of the fact that the amount of urine was in most subjects diminished. Two subjects, however, invariably showed low urine volumes during exercise, and in them the specific gravity was very high indeed. These two subjects (D and J) showed also the lowest urinary chloride outputs and the highest concentrations of chloride in sweat.

TABLE 3

Water output, intake, and balance for each subject, in 24-hour periods

	A	C	D	E	H	J	M	MEAN
Boulder City:								
Urine, cc.	1150	880	620	920	870	690	650	825
Evaporation in special exercise, grams	1130	2380	2490	1190	1220	1430	1760	1670
Drink, cc.	3210	4760	4305	3755	2830	4660	4410	3990
Body weight, grams	76,400	76,820	76,670	72,540	77,490	85,950	92,240	79,730
Daily weight difference, grams	338	337	498	421	345	500	233	382
Winter:								
Urine, cc.	1350	1110	950		1080		940	1090
Its specific gravity	1.018	1.029	1.027		1.020		1.032	1.025
Drink, cc.	890	1850	850		1250		2100	1390
Body weight, grams	75,620	77,800	77,530				90,740	80,420
Daily weight difference, grams	210	180	280				120	200

The rate of urine formation was much less during the stay in the desert than in winter climates (table 3), as has frequently been reported (Sundstroem, 1927). The lowest volume found in a 24-hour period was 440 cc. (in subject D). The concentration of the urine in the desert was considerably greater, while the total amounts of dissolved materials eliminated, particularly of chloride, appear to have remained unchanged.

Water intake. Throughout the period of life in Boulder City, each subject kept a record of all drink. The amount of food, and the water in it and formed from it, was not measured. Drink included milk, coffee, water and all other beverages. The average volume ingested per day (table 3) varied approximately according to the subjects' exposures to

evaporation; subjects C and D regularly took more exercise than others while subjects J and M were considerably larger in bulk and exercised moderately.

By this procedure the recorded fluid intake averaged 4000 cc. per day. In winter the same subjects ingested only 1400 cc. per day. It may be estimated that in both periods about 1000 cc. of additional water was contained in the food and formed in its oxidation; Magee (1937) computed that these sources furnished 760 cc. per day in sedentary men. Caspari and Schilling (1921) in a comparable African climate, but taking less exercise, found an average total intake of water of 3760 cc. per day. For a person in a winter climate, to drink 4000 cc. of fluids per day is a distinct discomfort. For a person in the desert to drink less is a violent discomfort.

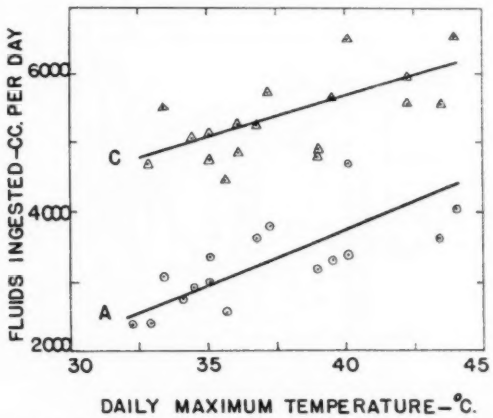

Fig. 1. Correlation of quantities drunk in 24 hours with maximum temperatures for the corresponding days. Subject C walked for two hours in the sun on each of these days, subject A for one hour.

Both forcing of intake and denial of intake are accompanied by impressive behaviors and sensations.

The highest intake recorded was 7370 cc. per day (subject D). During a previous expedition to Boulder City subject D drank 10,510 cc. on one day (Dill et al., 1933). Higher extremes of water ingestion have been measured by others, such as 11,400 cc. per day among steel mill workers (Dill et al., 1936) and 13,600 cc. per day among officers in India (Hunt, 1912).

The losses of water in urine, and in sweat during the formal exercise periods (table 3), account for more than half of the water turnover. The differences between the mean recorded drink and the mean recorded losses in these two categories were 700 to 2500 cc. per day. Evidently, the rate

of evaporation throughout the rest of the day was considerable, as already indicated in table 2.

For any one subject the variation in the amount of fluid taken was correlated markedly with the maximum temperature for the day (fig. 1). Under the regimes specified, subject A drank 160 cc. additional fluid for each rise of one degree C. in outdoor temperature, while subject C drank 120 cc. additional. With diverse types of exposure other increments would generally be required.

Throughout the periods of observation, body weight was measured upon arising each morning. The mean difference of body weight from one morning to the next varied (table 3) from 230 to 500 grams. This amount of fluctuation in body weight is only 8 per cent of the total amount of fluid turned over by the body each 24 hours. In other words, diverse

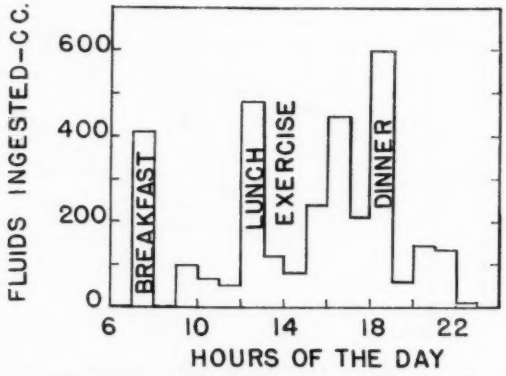

Fig. 2. Mean distribution by hours of *ad libitum* ingestions of fluid during 30 days in subject A.

subjects regained their initial water contents each day with a precision of 4 to 10 per cent of the amount of water exchanged.

The times of day at which most ingestion occurred (fig. 2) were *1*, immediately after exercise; *2*, with meals. It was the experience of some of the subjects that relatively little desire for water was felt *during* a period of 1 or 2 hours of exercise, yet during this period 1500 to 3000 grams of water were lost by evaporation.

In the subsequent recovery period only a part of this deficit of water was made up. Figure 3 indicates the amounts of water drunk by various subjects. These and similar curves show that satisfaction was obtained when only about half of the fluid lost had been restored. Several possible ways of viewing this result suggest themselves. It is possible that, since some solute as well as water was lost, only enough water was required by

the subject to render the concentration of the body fluids equal to their previous state. Another doubtful view is that the new content of the stomach or other portions of the alimentary tract is sufficient to allay thirst for the time being. It should be noted that the desire for water is assuaged before much of the water has been absorbed into the blood stream or any other tissue; and even after absorption has occurred only 50 to 70 per cent of the water deficit has been replaced within half an hour.

The form in which water was ingested seemed to make very little difference to the subjects. Most of the subjects preferred cold water to warm. Considerable satisfaction was found in taking the water in large draughts, as though the activity of the esophagus were an immediate part of the

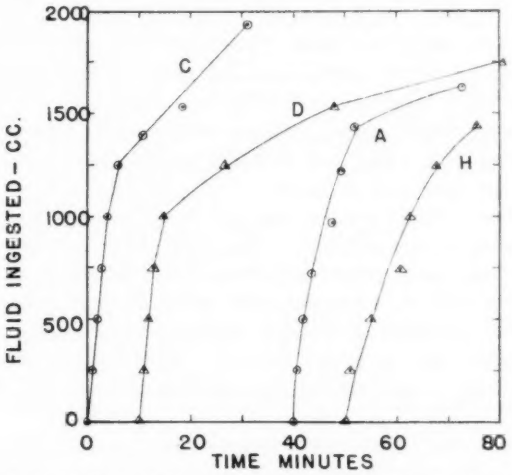

Fig. 3. Fluid ingested in relation to time, for each of four subjects on July 7th The fluid was cold grapefruit juice with sugar. Each subject had lost 2.0 to 2.7 kgm of weight by exercise within 3 hours, during which nothing was ingested.

assuagement of water lack. No uniform votes were obtained as to what tastes in the fluid taken were most desired (sugar, lemon, grapefruit, orange, root beer).

People in other climates are not accustomed to drinking water in quantities of half a liter or more at a draft. Men who travel in the desert or work in hot atmospheres habitually do not drink water as fast as they evaporate it, even when they have a plentiful supply. They make up the deficit at the end of the day (Chase, 1919; Dill et al., 1936). The replacement of water previously lost was equally slow when salts have not been at all depleted (Adolph, 1937).

A few attempts were made to anticipate the need for water before ex-

ercise was performed. In two experiments, a liter of water was taken within 30 minutes of the start of the exercise. In these cases very little of the fluid appeared as excess urine and most of it was apparently lost as perspiration. It is therefore possible to fill the alimentary tract, and probably some other tissues, with a certain amount of fluid which will be available for sweat. When, in two other experiments, the fluid was ingested an hour before the exercise, then most of it was lost in urine before it was used in perspiration. This observation also indicates that the function of the kidneys in reducing excess water contents of the body is as active as in other climates, even though there is rarely an opportunity for excess water to be present in the body. It may also be emphasized that none of the subjects showed a tendency to drink enough water at any one time to form urine faster than the usual rate, nor to form urine less concentrated than the usual. The only dilute urines found during the period (sp. gr. below 1.016) were the result of determined attempts to double the water intake on the part of two of the subjects.

On four days, dilute sodium chloride solution was ingested in place of water. In this form water was retained for several hours before it was required for the formation of sweat.

Water content. Body weight was not lost, but on the average was gained, by most of the subjects. The daily differences of body weight (table 3) were not much larger than those found in winter climates. This is the more remarkable since not only water content was varying with every period of exposure to outdoor conditions, but non-renal salt losses were much larger than in other climates. Then too, no effort was made to keep the intake of salt adequate or to control the intake of other food materials.

The maintenance of the body water content, therefore, depended upon the urges to ingest water which arose many times daily. Ingestion *before* water loss did not furnish a reserve on which the body later drew. All the deviations from water balance were on the negative side; water was expended and a debt built up, which was later paid off. Without entering into a discussion of the factors concerned in this urge to drink water—thirst—we wish to emphasize the accuracy with which thirst fulfills its function of securing water to make up all deficits.

Observations on a burro. For comparison with man it was decided to measure the rates of water intake and output on a desert animal, the burro. The individual studied during a period of 5 days was a female of ten months' age, weighing 97 kgm. Losses by evaporation (table 4) were at about the same rate during exercise as in man. This was in spite of the fact that the burro probably exposed a larger area of the body to direct solar radiation, and presumably produced about the same amount of heat while walking. In the first measurement (June 15) 3.5 kgm. were

lost in 2.9 hours. When offered water the animal drank 4.6 liters, which represented an intake in response to a deficit created throughout the previous 24 hours as well as during the exercise period. In the next 2 days of rest the water requirement of the burro was seen to be approximately 6 liters per day. Then an all-day experiment was undertaken in which 5 men, each for a period of 2 hours, walked with the burro. In the first 8 hours the burro lost 6.0 kgm. of weight while four men lost 5.9 kgm. Over the 10-hour period the burro lost 7.4 kgm. When now offered water, 12.2 liters were drunk within 10 minutes, and most of that amount within 5 minutes.

TABLE 4

Water balance in a burro (donkey)

DATE	HOUR	RECTAL TEMPERATURE	WALKED	BODY WEIGHT	WEIGHT LOSS RATE	WATER DRUNK	WATER INTAKE RATE
1937		*°C.*	*km.*	*kgm.*	*kgm. per hour*	*l.*	*l. per hour*
June 14	14.5						(drank)
	17.0		7				
June 15	13.67	38.4		97.15			
	16.60	38.6	9	93.65	1.19		
	16.75			Δ3.5		4.57	0.17
June 16	17.75					5.0	0.20
June 17	11.5					5.0	0.28
	17.5					1.75	0.29
June 18	8.25			95.45			
	10.27	38.5	6	93.95	0.74		
	12.28	38.2	6	92.55	0.70		
	14.08	38.95	4	91.17	0.76		
	16.15	39.0	6	89.47	0.82		
	18.42	39.63	7	88.05	0.62		
	18.58			Δ7.4		12.2	0.49
June 19	7.0	37.8		97.29		7.0	0.56
	13.0					2.0	0.33

The burro, like the dog (Adolph, 1937), takes an amount of water equal to its previous loss of water when water becomes available. This is the type of behavior suited to animals that experience intermittent availability of water. The human type of ingestion is unsuited to precarious water supplies. No other animal than man has been studied in which the amount of water taken at one time is insufficient to restore permanently the water balance. It is not known what factors of alimentary capacity or of rapidity of passage of water through the alimentary tract may be concerned in this difference.

SUMMARY

Modifications of water balances as measured during residence in the dry desert indicated that output by evaporation increased by 8-fold or more, while the concentration of sweat decreased. The output by urine remained constant or else decreased, while the urine concentration increased.

In exercise, rates of evaporation up to 1600 cc. per hour were regularly found, and urine rates down to 10 cc. per hour occurred at such times as the urine became chloride-free.

Intake of water was increased 3 to 6-fold and was accurately adjusted to previous losses of water by evaporation. Ingestion lagged considerably behind output during exercise and was largely made up at meals.

The rate of ingestion of water in man was slow in comparison with the burro, which drank water at a rate to restore its complete deficit within a few minutes.

The water content (weight) of the body was remarkably constant when measured at 24-hour intervals. The daily turnover of water was 10 to 30 times the daily variation in body weight.

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HUNGER AS A DETERMINANT OF CONDITIONAL AND UNCONDITIONAL SALIVARY RESPONSE MAGNITUDE

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It is well known that the magnitude of any conditional or unconditional salivary response is determined by several factors. Some of these determinants have been studied systematically, others are rigidly controlled but have not been subjected to experimental investigation. This experiment purposed to investigate the conditional and unconditional response rôle of a recognized and well-controlled but relatively unstudied variable: hunger.

The six dogs used as subjects were selected because of their previous conditional response (CR) experience. Each had a satisfactory parotid fistula and all had relatively stable, active CR's.

The unconditional stimulus (UCS) in every case consisted of the mechanical presentation of two dog biscuits. The conditional stimulus (CS) was acoustic for every subject: sound of doorbell (animals 1, 2, 3, 4), electric sound hammer one beat per second (no. 5), and sound of air bubbling through water (no. 6). For purposes of this experiment, identical CS's (for all subjects) are obviously not requisite since all comparisons are of the responses of individual subjects under a single varied condition.

In each daily experimental session, ten CS-UCS combinations were presented to each subject with varied temporal periods between presentations. A CS-UCS combination consisted of CS 15 seconds, food presented ten seconds after beginning of CS. The CR was recorded as the volume of saliva secreted during the first ten seconds after the beginning of the CS. The unconditional response (UCR) was recorded as the amount of saliva secreted during the sixty seconds after food presentation. It may be noted that comparison of the reliability of the conventional technique of recording the CR (saliva secreted during 30 to 60 seconds) with the technique used in the present experiment demonstrates little superiority in

[1] Yale Laboratories of Primate Biology. This investigation was conducted while the writer was a National Research Council Fellow, Division of the Biological Sciences. Grateful acknowledgment is made to Dr. W. H. Gantt for his counsel throughout the experiment.

the usual experiment for the longer recording. The present technique has three advantages: 1, experimental extinction cannot influence the results, even though several stimuli are presented, since reinforcement by UCS occurs each time; 2, more experimental trials per session may be presented; and 3, the possible formation of delayed CR is eliminated. The recorded UCR, of necessity, includes a CR component since CS temporally overlaps UCS. Apparatus and recording instruments used have been described elsewhere (3).

Each animal was tested through two identical series of food-deprivation periods; each series included fasts of 0-hour (satiation on dog biscuits immediately before experiment), 24-, 48-, 72-, and 96-hours. Sufficient time was allowed to elapse between series (with the animals fed their regular diets) to insure return to normal nutritive condition at the beginning of the second series.

TABLE 1

ANIMAL NUMBER	HOURS FOOD DEPRIVATION									
	0		24		48		72		96	
	CR	UCR	CR	UCR	CR	UCR	CR	UCR	CR	UCR
1	0.01	0.36	0.35	1.95	0.34	1.82	0.40	1.38	0.38	1.60
2	0.06	0.73	0.09	0.61	0.15	0.65	0.17	0.78	0.14	0.54
3	0.06	0.94	0.14	0.73	0.16	0.76	0.16	0.94	0.02	0.60
4	0.04	1.44	0.18	0.72	0.23	0.58	0.25	0.90	0.22	0.54
5	0.01	0.12	0.13	1.82	0.15	1.44	0.17	1.55	0.12	1.10
6	0.08	1.44	0.10	0.82	0.15	0.81	0.24	1.08	0.12	0.72

Table 1 presents the mean absolute CR and UCR magnitudes in milliliters for each of the subjects. When the CR values are ranked according to size for each subject and mean ranks computed, the rank from smallest to largest is: 1, 0-hour; 2, 24-hours; 3, 96-hours; 4, 48-hours; 5, 72-hours. In the same way UCR ranks are: 1, 96-hours; 2, 24-hours; 3, 0-hour; 4, 48-hours; 5, 72-hours. However, this crude ranking cannot be accepted at its face-value since the relative magnitudes of both CR and UCR for the different subjects are highly variable. This variability and the variability of the individual subjects' readings unquestionably distorts the true picture of the effects of food deprivation. In order to correct this distortion, means for each of the deprivation periods were computed from the individual subjects' means weighted in accordance with the generally accepted principle that the reliability of a mean is proportional to the reciprocal of the square of its standard error (cf. 2, p. 55).

Figure 1 presents the weighted CR and UCR means for each deprivation

period.[2] Inspection of these means indicates that the same empirical equation might satisfactorily describe both the CR and the UCR data. Simple parabolic curves ($y = a + bX + cX^2$) were fitted by the method of least squares to both sets of data; determination of goodness of fit showed that the data are adequately described by these curves. While the fundamental identity of the factors determining relative CR and UCR magnitudes is not demonstrated by these data, food-deprivation *per se* stands out so strongly as a determinant that the effects of any particular determinants are obscured.

The data as presented demonstrate conclusively the significant effects of food-deprivation; unquestionably an accumulation of more data gathered

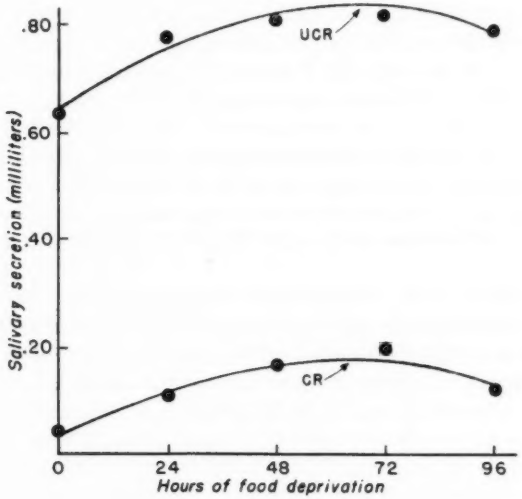

Fig. 1

from the populations here sampled would result in an increased reliability. The true effects of food-deprivation on CR and UCR magnitudes would then stand out without the necessity of clarifying the data with statistical manipulation as above. That an increase in the number of observations is desirable is, however, open to question since the long-time effects of

[2] The values presented are computed from the formula;

$$wM_t = \frac{\frac{M_a}{\sigma_a^2} + \frac{M_b}{\sigma_b^2} \cdots + \frac{M_n}{\sigma_n^2}}{\frac{1}{\sigma_a^2} + \frac{1}{\sigma_b^2} \cdots + \frac{1}{\sigma_n^2}}$$

repeated food-deprivation periods—even when interspersed with normal diet days—might be such as to render the animal unfit for further experimental observation.

The fact that CR and UCR magnitudes are reported above for 0-hour deprivation seems to demand further explanation. If an animal is *completely* satiated, does that not mean that he will refuse proffered food? And if he refuses food, how can UCS be applied? If UCS cannot be applied, how can UCR be elicited? In every case of 0-hour deprivation, the subjects were fed until they no longer evinced interest in food. They were then coaxed (usually successfully) to eat more. Finally food was placed in their mouths until they began actively to reject it. Upon being placed in the test room, the animals would, in the early part of the period, sometimes refuse to eat when UCS was presented. Such refusals were rare. In every instance, however, CR was elicited—sometimes of a very small volume. In no case did a subject consistently reject UCS. Two explanations may be offered for this acceptance of food in the test situation when the same food was refused outside of that situation: 1, some time (a matter of a few minutes) must elapse between the feeding and testing; perhaps this elapsed time is sufficient to allow the satiation effects to wear off; and 2, the animal's habitual mode of response (i.e., food-taking following secondary stimulation) may be so firmly established that it outweighs the satiation effects.

The coincidence of the elicitation of minimal CR and UCR at 0-hour deprivation (food-satiation, minimal hunger) and of maximal CR and UCR during the 72-hour deprivation period (when, it may be conjectured from the work of Carlson (1), hunger is probably nearly maximal), demonstrates the necessity for taking motivational factors into account in CR study.

CONCLUSIONS

1. Food deprivation affects both the conditional and unconditional salivary responses in relatively the same way: volume of salivary secretion increases from minimal at 0-hour's deprivation (food-satiation) to maximal at 72-hours; at 96-hours the volume of secretion falls to near the 24-hour level.

2. The coincidence of minimal secretion with minimal hunger and of maximal secretion with near maximal hunger indicates that the strength of the "drive" stimulus is an important determinant of CR and UCR magnitude.

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THE EFFECT OF ALTERING THE RENAL BLOOD PRESSURE AND BLOOD FLOW ON THE GLOMERULAR FILTRATION OF A TRANSPLANTED KIDNEY IN UNANESTHETIZED DOGS[1]

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The relation of the renal blood pressure and blood flow to the creatinine clearance has recently been studied by Medes and Bellis (1934) and Medes and Herrick (1933). The latter authors, using the thermostromuhr of Rein, have shown that the creatinine clearance generally parallels the blood flow of the kidney. Medes and Bellis determined the glomerular filtration in anesthetized dogs following reduction of the renal blood pressure by partial occlusion of the aorta above the renal arteries. They concluded that the glomerular filtration as determined by the creatinine clearance test directly parallels the renal blood pressure. Similar findings were obtained by Lassen and Husfeldt (1934) in patients with a blood pressure lowered by the administration of spinal anesthesia.

The method to be described for transplanting a kidney distal to a carotid loop permits alteration of the renal blood pressure in unanesthetized dogs. Repeated studies of the glomerular filtration can be made. Measurement of the renal blood flow can also be carried out without general anesthesia.

That the function of a transplanted kidney may be essentially normal has been shown by numerous investigators (Quinby, 1916; Ibuka, 1926; Holloway, 1926). Herrick, Essex and Baldes (1932) measured the blood flow of a transplanted kidney with the thermostromuhr and state: "The flow of blood to the transplanted kidney seems to be about that of the intact kidney. Glucose, sodium sulphate and epinephrine have the same effect on the flow of blood to the transplanted kidney as they have on the normal kidney." Furthermore, life has been supported for months by a single kidney transplanted to the neck (Ibuka).

The present publication deals with a study of the effects of constriction of the artery to a transplanted kidney on the renal blood pressure, blood flow and creatinine clearance. In addition, the glomerular filtration of

[1] Aided by a grant to Vanderbilt University by the Division of Medical Sciences of the Rockefeller Foundation.

[2] National Research Fellow in the Medical Sciences.

a transplanted kidney was compared to that of the intact kidney in a few experiments.

METHOD. Dogs were used in all experiments. At a preliminary operation the left carotid artery was enclosed in a tube of skin by Van Leersum's method. After the wound had completely healed, the left kidney was transplanted to a pocket under the skin of the neck. The carotid artery distal to the loop was anastomosed to the renal artery and the external jugular vein to the renal vein by the method of Carrel. The ureter was brought out through a small opening in the skin. A photograph of an animal with this preparation is shown in figure 1. The non-transplanted kidney was removed a few days later.

Food was withheld for 20 hours preceding the studies. Creatinine in a dosage of 0.1 gram per kilo was administered intravenously one hour before starting the first period. At the same time 250 cc. of water were given by stomach tube. The transplanted kidney was sometimes cath-

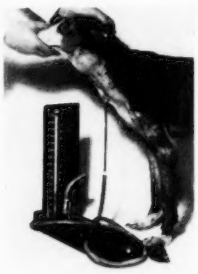

Fig. 1. Showing Van Leersum loop and transplanted kidney distal to it.

eterized for the duration of the experiment but usually was not since infection is apt to occur following such a procedure. The urine remaining at the end of each period was expressed by gentle pressure on the kidney pelvis. Blood samples were drawn from the femoral artery at the middle of each period. The amounts of creatinine in the urine and blood plasma were determined by the methods used by Van Slyke, Hiller and Miller (1935) except that the usual tungstic acid blood filtrate was employed for the determination of creatinine in the blood. The glomerular filtration per minute was obtained from the product of the concentration index, C, and the flow of urine per minute (Rehberg, 1926).

$$C = Cr\ U/Cr\ Pl$$

where $Cr\ U$ and $Cr\ Pl$ represent the concentration of creatinine in the urine and plasma respectively. Then

$$C \times \frac{\text{volume of urine}}{\text{time in minutes}} = \text{filtration per minute.}$$

Blood pressure determinations were made in all instances without anesthesia by inserting into the lumen of the femoral artery and carotid artery distal to the constriction a needle connected to a mercury manometer.

Several methods were used to compress the carotid artery supplying the transplanted kidney. In some experiments a cuff with an inflatable rubber bag was wrapped around the proximal end of the carotid loop and pressure in the cuff regulated with a bulb and manometer (fig. 1). In other experiments a Goldblatt clamp[3] was placed directly on the carotid artery at the proximal end of the loop under local anesthesia. In a few experiments a rubber band or a screw clamp was applied to the carotid loop but these methods were less satisfactory.

Regulation of the renal blood pressure by partial constriction of the artery to a transplanted kidney is quite difficult because the blood pressure tends to return spontaneously to the normal level following moderate compression of the carotid artery. This disadvantage was overcome to some extent by making frequent determinations of the renal blood pressure and increasing the constriction occasionally to maintain the lowered level of the blood pressure.

The blood flow of the transplanted kidney was determined by inserting a cannula into a lateral opening in the external jugular vein through which the flow could be diverted for a short period. A small amount of novocaine was previously injected into the skin overlying the vein. Heparin was used as the anti-coagulant.

Results. Ten experiments were performed on three dogs and the results of nine are given in table 1. One experiment is omitted because the blood samples were lost. Glomerular filtration studies were carried out in seven of the tabulated experiments. A reduction in the renal blood pressure by constriction of the artery to the transplanted kidney caused a parallel reduction in the renal blood flow in the three experiments in which the blood flow was measured. A spontaneous rise in the renal blood pressure after constriction had been present for a short time was accompanied by a parallel increase in the blood flow in these experiments. This relationship is apparently constant under the conditions of these studies.

Vasoconstriction in the kidney appears not to be the factor responsible for the spontaneous rise in the blood pressure following partial occlusion of the artery to the transplanted kidney because the alterations in blood flow parallel the changes in blood pressure. Possibly the degree of constriction is gradually diminished as a result of thinning of the wall of the artery at the site of the clamp on the artery.

A reduction in renal blood pressure and flow was followed by an immediate decrease in the glomerular filtration in all experiments. The results

[3] Clamps were obtained through the courtesy of Dr. Harry Goldblatt.

TABLE 1

*The effects of constricting the artery to a transplanted kidney on the renal blood pressure, blood flow and glomerular filtration**

DOG NUMBER	DATE	EXPERIMENTS	NUMBER PERIODS	LENGTH PERIODS	URINE VOLUME	CREATININE		CONCENTRATION INDEX	GLOMERULAR FILTRATION	ARTERIAL BLOOD PRESSURE		RENAL FLOW	METHOD OF CONSTRICTION OF ARTERY
						Urine	Plasma			Renal	Femoral		
				min.	*cc.*	*mgm. per cent*	*mgm. per cent*		*cc. per min.*	*mm. Hg*	*mm. Hg*	*cc. per min.*	
1	9/29	Control	4	15	29.7	194	9.84	19.71	38.56		166		Large screw clamp to loop
		Constriction	1	15	8.9	161	8.34	19.35	11.48	80			
		Release	3	15	26.5	153	8.34	18.26	29.55	130			
	10/ 4	Control	4	15	39.1	95	21.9	4.33	10.82	120	174		Goldblatt clamp to artery
		Constriction	3	15	2.5	113	20.6	2.13	5.46	45			
		Release	1	15	25.2	79	20.7	3.71	6.23		180		
	10/29	Control	4	15	19.5	225	15.55	14.5	18.7		150		Rubber band applied to loop
		Constriction	3	28	2.4	207	16.40	11.5	3.6	82	160		
		Release	3	15	9.3	231	15.28	15.4	9.5	145	142		
	11/ 3	Control	3	15	32.7	145	11.92	13.3	29.0		155		
		Constriction	2	18	4.5	156	11.17	17.2	5.2	70	150		
		Release	3	15	25.8	138	11.03	12.7	21.6	138			
2	11/ 5	Control	3	15	25.9	179	11.86	15.4	17.6	120			Inflatable cuff to loop
		Constriction	1	14	6.7	203	11.40	17.8	8.5	93	155		
		Release	4	15	16.6	197	9.81	20.6	22.5				
	11/13	Control	3	15	31.9	347	15.39	22.52	47.5	140	145		Goldblatt clamp applied directly to artery
		Constriction	5	15	9.9	398	15.97	24.92	16.0	96	155		
	11/19	Control	4	15	24.5	380	22.6	17.3	26.8	121	125	297	
		Constriction	8	15	8.0	571	19.79	29.6	15.9	97	137	230	
3	12/22	Control								167	170	133	Spontaneous changes in renal blood pressure and flow following constriction with Goldblatt clamp
		Minutes duration of constriction											
		1½								25		29	
		12										80	
		19								63		74	
		34										80	
		52								145	152	135	
	12/23	Control								120	150	74	
		Minutes constriction											
		1								63	175	38	
		11								55		50	
		26								90	160		

* Where observations were made for more than one period the average of the determinations is tabulated.

agree with those that Medes and Bellis and Medes and Herrick obtained using animals with a non-transplanted kidney.

In four experiments the glomerular filtration of a transplant and a normal kidney were determined simultaneously. The results will not be given in detail because some degree of ureteral obstruction was present in all of the transplants. In one dog with slight ureteral obstruction, the glomerular filtration of the transplant was 69 per cent of the filtration of the normal kidney.

SUMMARY

A method which permits a study of experimental alterations in renal function in unanesthetized dogs has been described. It consists of transplanting a kidney distal to a Van Leersum loop, the renal artery being anastomosed to the carotid which had been placed previously in a tube of skin. The decline in blood pressure which followed constriction of the artery to the transplant was accompanied by parallel changes in the renal blood flow and glomerular filtration.

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RECOVERY CYCLE OF MOTONEURONS[1]

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In a previous publication (Lorente de Nó, 1935b) it was reported that the ocular motoneurons recover from the refractoriness created by an antidromic impulse through a phase of depressed excitability lasting considerably longer than the classical relatively refractory period in nerve. Since the oculomotor nerve was found to recover through a similar cycle, and it was thought at the time that the supernormal phase occurs in the recovery of excised nerve only, the conclusion was reached that the long-lasting refractoriness of the motoneurons was comparable to the subnormal phase of peripheral nerve (Graham, 1933, 1935; Gasser, 1935a). This conclusion was of far-reaching significance, because it indicated that subnormality which, as Gasser had demonstrated, increases with tetanic activity, might play an important rôle in the production of inhibition (Gasser, 1935b, 1937a, b, c) and that the slowing of the recovery process by summation of subnormality might lead to cessation of the rhythmic activity of chains of neurons, including closed loops (Lorente de Nó, 1935c, 1936).

When later it became known that blood-perfused nerves often manifest supernormality (Lorente de Nó and Graham, 1935–36; cf. Gasser and Grundfest, 1936; Graham and Lorente de Nó, 1938), it was necessary to determine whether simultaneously with the occurrence of such supernormality in the recovery of motor fibers, the motor somata also manifest supernormality. Contrary to expectation, it was consistently found that motoneurons never develop supernormality for synaptic stimuli, not even when their axons show a pronounced supernormal phase for electrical stimuli. Direct comparison between the late part of the recovery of motoneurons and the subnormal phase in nerve was, therefore, no longer possible, and it was necessary to investigate the effect of rhythmic activity on the recov-

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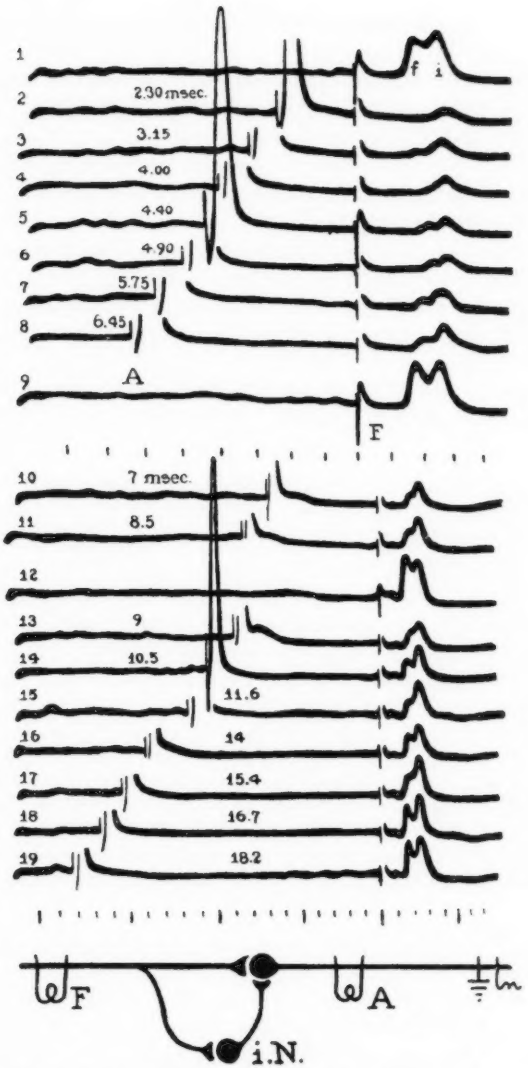

Fig. 1. Oculomotor preparation of the rabbit. Responses recorded from the trochlear nerve (1-21-36). Position of the electrodes indicated in the diagram at the bottom. The records illustrate the recovery of a synaptic response elicited by a shock through the *F* electrodes, after delivery of a maximal antidromic shock through electrodes *A*. The synaptic response is composed of two waves, *f* and *i*, the second caused by impulses delayed at an internuncial neuron i.N. Time given in msec. underneath records 9 and 19 applies to the records above. Intervals between the *A* and *F* shocks indicated in msec. on each record. Approximate conduction time for the *A* impulses to the motor nucleus, 0.4 msec.; for the *F* impulses, 0.15 to 0.20 msec.

ery of motoneurons. Unless this recovery, like that of axons, was depressed by rhythmic activity, the explanation of inhibition in terms of subnormality would not be valid.

TECHNIQUE. The observations reported in the present paper were made on the oculomotor preparation of the rabbit previously described (Lorente de Nó, 1935a, b). Refractoriness of motoneurons was created by antidromic impulses started in their axons by maximal shocks delivered to the oculomotor nerve shortly after it leaves the brain-stem. The testing response was produced by a shock delivered to the posterior longitudinal bundle and adjacent pathways at the floor of the fourth ventricle. The

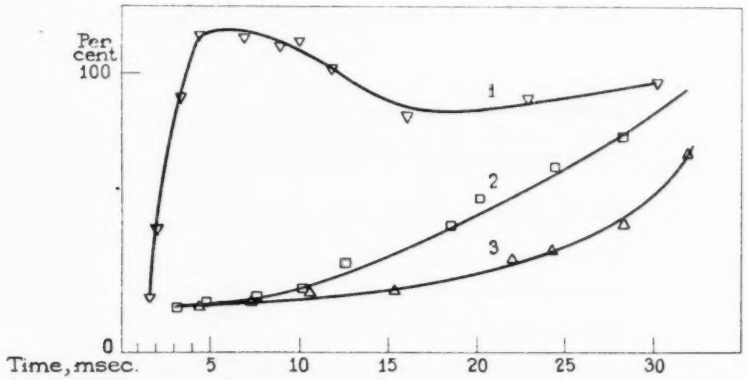

Fig. 2. Oculomotor preparation; responses recorded from the internal rectus muscle (6-12-36). Stimulating electrodes as in figure 1. The curves are plots of height of testing response (ordinates) against intervals between shocks (abscissae). 1. Recovery curve of the oculomotor nerve, one maximal *A* response conditioning one submaximal *A* response. 2. Recovery curve of the synaptic response after delivery of one maximal conditioning *A* shock. 3. Recovery curve of the synaptic response conditioned by a series of three antidromic shocks at the frequency of 100 per second, the conditioning interval being measured from the last antidromic shock.

impulses thus produced are conducted directly to synapses on ocular motoneurons, which after the synaptic delay respond by firing impulses into their axons. The responses were recorded after conduction from the internal rectus muscle or the trochlear nerve.

RESULTS. *Recovery of motoneurons after one response.* Two typical experiments are illustrated in figures 1 and 2. In both the recovery of electrical excitability of the axon was of type *A* (Graham and Lorente de Nó, 1938, fig. 1), and showed four phases—absolutely refractory period, relatively refractory period, supernormal period, and subnormal period. The recovery of the motoneurons was essentially the same in both experi-

ments; but in contrast to the recovery of the axons, it included simply a long period of depressed excitability (curve 2, fig. 2; fig. 1). This depression ended at about the same time as did the subnormal period of the axonal recovery.

The characteristic slow recovery of synaptic excitability may be studied in detail in the tracings of records included in figure 1. The testing response was composed of two waves, the first (*f*) caused by motoneurons responding to impulses delivered to the motor pool by the posterior longitudinal bundle, and the second (*i*) with its additional latency of about 0.6 msec. attributable to other motoneurons responding to impulses delayed by passage through one internuncial neuron. The earliest response, which was obtained at an interval of 2.3 msec. between shocks, consisted of a small *i* wave only (record 2). The *f* wave appeared when the interval was somewhat longer than this, but it did not approach its unconditioned size until the interval was 17 to 18 msec. Obviously, at short intervals between shocks some motoneurons contributing to the unconditioned *f* wave did not respond to the impulses delivered by the posterior longitudinal bundle; but they did respond to internuncial impulses arriving 0.6 to 0.9 msec. later, and thus contributed to the *i* wave. The persistently high threshold of the motoneurons is indicated not only by the decreased height of response throughout the recovery period, but also by the longer latency manifested in records 2 through 8 on a fast time axis (lenthening of the synaptic delay during refractoriness; cf. Eccles, 1936a, b, 1937).

The contrast between the recovery of the motoneurons and of their axons is particularly striking when, as in the experiments chosen for illustration, the excitability of the axons is actually supernormal at certain intervals. But even though actual supernormality may be absent, the recovery of the axons is always more rapid than that of the motoneurons, and they ordinarily manifest a peak of excitability which it has been impossible to duplicate convincingly in the case of the motoneurons. If a peak such as this exists, its magnitude must be below that clearly demonstrable under the conditions of the present experiments. Therefore, curve 2 of figure 1 and the curves previously published (Lorente de Nó, 1935b, fig. 2) will be considered typical of the recovery of excitability of the motor nucleus.

In the recovery cycle of sympathetic ganglion cells after the delivery of an antidromic impulse Eccles (1936a) found a peak of excitability in a long-lasting period of depression. Similar results were reported by Eccles and Pritchard (1937) for spinal motoneurons. It is possible that certain factors operative in the sympathetic ganglion and in the cord are absent or ineffective in the oculomotor preparation.

A simple explanation of the difference between the recovery cycles of

the motoneurons and their axons would be that the supernormality of electrical excitability is an artefact attributable to local change of the nerve at the level of the stimulating electrodes. But this explanation is conclusively excluded by control experiments such as that illustrated by figure 3, II, in which the conditioning response was started by presynaptic stimulation. It is obvious that the passage of impulses started

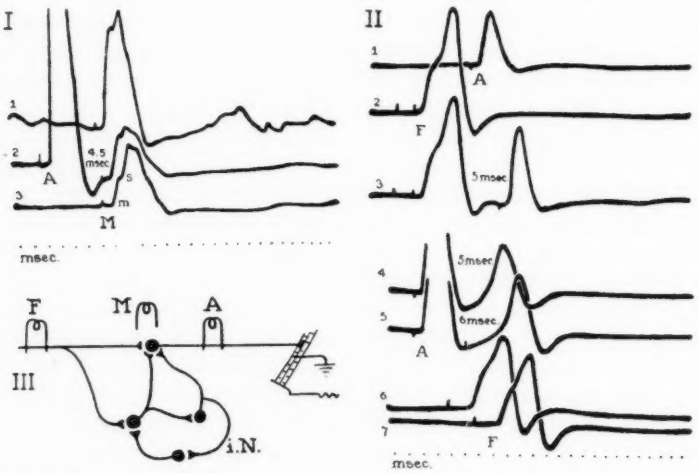

Fig. 3. Oculomotor preparation; responses recorded from the internal rectus muscle. Position of the stimulating electrodes indicated in III.

I. (10-25-35). One shock through the *M* electrodes sets up a response including two waves, *m* attributable to motoneurons directly stimulated by the shock, and *s* to motoneurons stimulated by internuncial impulses (record 3). Intercurrent internuncial discharge greatly increases the *m* wave (record 1). A shock through the *A* electrodes also increases the *m* wave (record 2) because the motoneurons acquire supernormal excitability for electrical stimuli. Time in msec. Interval between the *A* and the *M* shocks indicated on record 2.

II. (10-23-35). A large synaptic response to two *F* shocks in quick succession (record 2) causes a submaximal *A* response (record 1) to acquire supernormal height (record 3); while a maximal *A* response (not reproduced in totality) reduces the response to one *F* shock (records 6 and 7) to subnormal size (records 4 and 5). Approximate interval between responses indicated in msec. in records 3, 4 and 5.

at the somata of the motoneurons by an entirely physiological process created supernormal excitability in the nerve, although the entrance into the somata of antidromic impulses after about the same interval set up there subnormal excitability for synaptic stimuli.

Experimental evidence is also not in conformity with the hypothesis that the difference between the recovery of synaptic excitability of the soma and the recovery of electrical excitability of the axon depends upon

a qualitative difference between soma and axon, for by the experiment next described it may be shown that the soma resembles the axon in its response to electrical stimulation.

Shielded stimulating electrodes are buried in the anterior colliculus so that their free points reach the reticular substance in front of the oculomotor nucleus, and a stimulus is applied through them. Part of the response obtained is caused by electrical stimulation of the somata of motoneurons (Lorente de Nó, 1935d), and this part shows a phase of supernormal excitability after delivery of an antidromic impulse. Figure 3, I, reproduces records obtained in an experiment of this kind. The response to a single shock applied to the reticular substance (record 3) consisted of two waves, *m* and *s*. The *m* wave had a latency equal to the time required for the conduction of the impulses from the motor nucleus to the muscle plus the end-plate delay, while the *s* wave had an additional latency of about 0.7 msec. (0.7 msec. is the average synaptic delay of impulses delivered at the synapses of the motoneurons). Undoubtedly, the *s* wave resulted from responses of some of the motoneurons to internuncial impulses started by the shock, and the *m* wave from direct electrical stimulation of other motoneurons. That this direct stimulation affected the somata is demonstrated by the enormous increase of the *m* response when the tonic innervation of the eye muscles was increased and irregular waves appeared in the electrogram of the muscle (record 1). The *m* response became larger, because the additional impulses which were being delivered to the motoneurons lowered their electrical threshold, an effect that can occur only where synapses are located, i.e., on the somata. Delivery of a conditioning antidromic shock (record 2) at a suitable interval before the testing shock made the *m* response larger than in the normal, unconditioned response (record 3). The conclusion drawn from these results is that the antidromic impulse had created supernormal excitability for electrical stimuli in the somata of the motoneurons.

One objection that may be raised against this experiment is that during the supernormal period created by the passage of the antidromic impulse the electrical threshold of the axons might become lower than that of the somata, and that consequently the increase in the *m* wave might be caused by responses started in the axons of some of the motoneurons simultaneously with responses from the somata of other motoneurons. But this objection was met by other experiments in which the testing response was elicited by the successive delivery of two shocks, one to the posterior longitudinal bundle in the floor of the fourth ventricle and the other to the motor nucleus. A number of motoneurons responded only to this combined stimulation, and there can be no doubt that their responses are to be attributed to electrical stimulation of the somata. Of the three experiments carried out in this manner the motor nerve developed supernormal-

ity in only one; in the other two the early rise of the recovery curve of the nerve response did not reach the normal level (type C of recovery, Graham and Lorente de Nó, 1938, fig. 1). In all three cases the response of the somata to the combined stimulus recovered promptly, in conformity with the recovery of the axonal response, and in marked contrast to the slow recovery of a response initiated by synaptic stimulation alone.

Thus the conclusion is unavoidable that the recovery cycle of the motoneurons when tested with electric shocks differs from that when it is tested with synaptic stimuli, chiefly because supernormality to electrical shocks is not accompanied by increased synaptic excitability. However, lack of information on the mechanism of synaptic transmission makes it impossible to offer a satisfactory explanation of this difference.

Recovery of motoneurons after tetanic activity. The question as to whether rhythmic activity delays the recovery of the response of motoneurons to synaptic stimuli, as it delays the recovery of electrical excitability of nerve (Gasser, 1935a; Gasser and Grundfest, 1936; Graham and Lorente de Nó, 1938), was answered in the affirmative by experiments in which recovery of synaptic response after one antidromic conditioning response was compared with recovery after a series of two or more such conditioning responses. In view of the reports published by Eccles and Hoff (1932) and by Hoff, Hoff and Sheehan (1934), this result was to be expected, although these authors did not correlate the deficit of response with a rise in the threshold of the motoneurons (cf. Gasser, 1935b, 1937a; Eccles, 1936b).

Our results are illustrated by curves 2 and 3 of figure 2, and by figures 4, 5 and 6. In figure 2, the recovery of synaptic excitability after one conditioning impulse is compared with the recovery after three impulses at a frequency of 100 per second. The slowing of recovery, i.e., summation of subnormality, after rhythmic activity is clearly shown.

In the experiment from which the records of figure 4 were taken, a synaptic testing response (1c, 2c, 3c, 4c) was conditioned 1, by a series of 7 antidromic shocks at a frequency of 100 per second, ending 30 msec. before the testing response (1d, 2d, 3d, 4d); 2, by a single antidromic shock at various intervals before the testing response, responses at 3, 5, 10 and 18 msec. intervals having been chosen for illustration (1b, 2b, 3b, 4b); or 3, by the series ending at 30 msec. plus the single shock at the same four intervals (1a, 2a, 3a, 4a). At every interval the response conditioned by the combination of the series and the single shock was smaller than when it was conditioned by either the series or the shock alone. This is particulary striking in the lowest set of records, where the depression produced by the single shock at 18 msec. or that produced by the series at 30 msec. is slight; but the depression produced by the single shock and the series combined is much more marked.

In other experiments similar to this, but having somewhat more frequent series, the introduction of the single shock after the series did not appre-

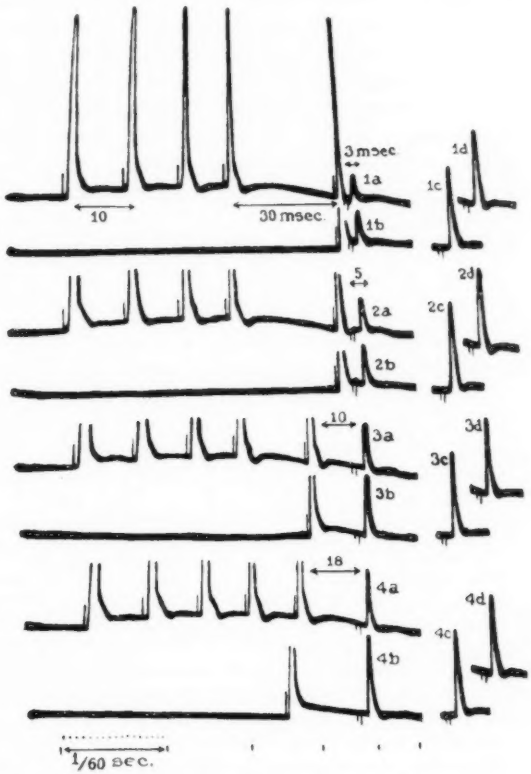

Fig. 4. Oculomotor preparation; responses recorded from the internal rectus muscle (6-12-36). Stimulating electrodes as in figure 1. Synaptic response: unconditioned, 1c, 2c, etc.; conditioned by single antidromic shock, 1b, 2b, etc.; conditioned by a series of 7 antidromic shocks at a frequency of 100 per second, 1d, 2d, etc.; conditioned by both the single shock and the series, 1a, 2a, etc. Intervals between the different responses indicated on the records; interval between last response of the series and the synaptic response, 30 msec. throughout. Time in thousandths and sixtieths of a second. Irregularities in the thyratron circuit controlling the start of the sweep caused the responses to appear at different positions in the oscillograph, although the intervals between shocks, which were controlled by a rotary interrupter, remained constant for each one of the four settings.

ciably increase subnormality. It will be noted in figure 2 that rhythmic activity at short intervals does not produce greater depression than does one conditioning response, i.e., under conditions in which the de-

pression reaches a certain limit, additional activity apparently does not increase it further. The number of experiments has not been sufficient to determine accurately the limiting amount of depression, but it seems to be the amount existing 1.5 to 2 msec. after one response.

There can be no doubt that rhythmic stimulation within certain limits causes depression of synaptic excitability be means of an additive process, and it is interesting to determine the relation of the frequency of responses to the increase of subnormality. An approximate quantitative relationship may be derived from the observations plotted in figure 5 (cf. original records in fig. 6). The synaptic testing response was conditioned by a series of two antidromic shocks. The interval between the second antidromic and the testing response was maintained constant, but the interval

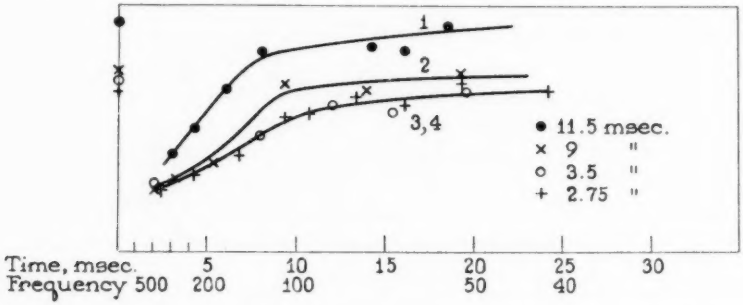

Fig. 5. Oculomotor preparation; responses recorded from the internal rectus muscle (6-12-36). Stimulating electrodes as in figure 1. Synaptic response conditioned by two maximal antidromic shocks at variable intervals. Interval between the second antidromic and the testing response constant (2.75 msec. for curve *4*; 3.5 msec. for *3*; 9 msec. for *2*; 11.5 msec. for *1*). Height of synaptic response (ordinates) plotted against frequency of the conditioning shocks (abscissae). Heights of responses conditioned by the second antidromic shock only shown on the ordinate axis.

between the antidromic shocks was varied from 2 to 25 msec., i.e., the frequency was varied from 500 to 40 responses per second. Determinations were made at four intervals—2.75, 3.5, 5 and 11.5 msec.—between the second antidromic shock and the testing responses. The heights of the testing responses, conditioned only by the second antidromic shock, are indicated on the ordinate axis, while the heights of the responses conditioned by both antidromic shocks are plotted in curves 1 to 4 against frequency of the series. Evidently the decrease in ordinate values measures the increase of subnormality.

It appears that for frequencies below 40-50 per second the summation of subnormality is scarcely noticeable. But frequencies higher than 50 per second cause a marked increase in subnormality, which is greater,

the higher the frequency. Each of the four curves has two parts with different slopes meeting at about the abscissa value of 100. This justifies the statement that for frequencies between 50 and 100 per second the increase in subnormality is rather small, but for frequencies above 100 per second subnormality grows rapidly, almost in linear relation to frequency. Since the curves of figure 5 converge at the left toward the same point, it is evident that at this frequency, i.e., 500 per second, the subnormality produced by two discharges persists almost unchanged for a considerable period of time after the second discharge. The initial flat

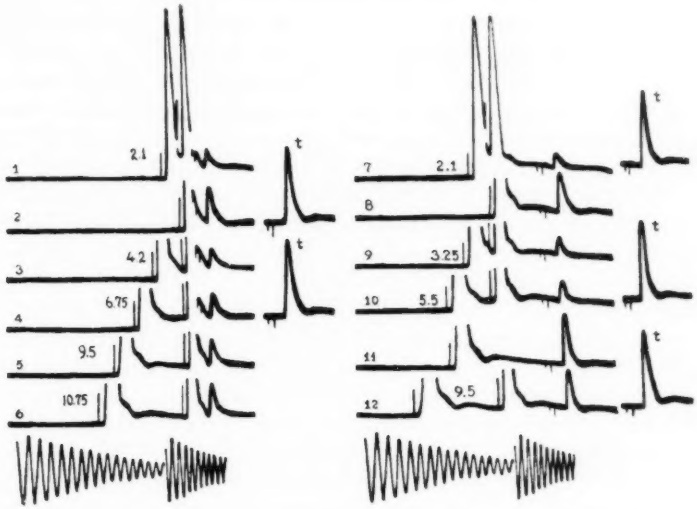

Fig. 6. Some of records from which curves 2 and 4 of figure 5 were constructed. Records marked t, unconditioned synaptic response; records 2, 8 and 11, response conditioned by one antidromic shock; other records, response conditioned by two antidromic shocks. Intervals between antidromic shocks indicated on the records; interval between the second antidromic and the *F* shock, 2.75 msec. for records 1 through 6, 9 msec. for records 7 through 12. Time, 1000 cycles.

segment of curve 3 in figure 2 illustrates a similar phenomenon after several shocks at lower frequencies.

In the present study, rhythmic activity of motoneurons has been produced with relatively short series of antidromic impulses, up to 10 shocks, and consequently there was no opportunity to observe the phenomena recently described by Kleyntjens (1937) after prolonged tetanization of spinal motor roots in the frog.

Discussion. The apparent failure of rhythmic activity to increase the amount of depression obtained within 1.5 to 2 msec. after a response, and the marked effect of such activity in increasing the depression at

greater intervals, suggests a division of the recovery cycle of motoneurons into two parts. During the first part, the course of recovery is relatively fixed, or at least is but little affected by rhythmic activity; while the further course of recovery, during the second part, is greatly delayed by rhythmic activity at frequencies above a certain level. This division of the recovery cycle of motoneurons may be compared to the division suggested for the recovery cycle of peripheral nerves, into an early phase, also lasting for 1.5 to 2 msec., which is not appreciably affected by rhythmic activity, and a late phase easily depressed by tetanization (Graham and Lorente de Nó, 1938). But the comparison between the two recovery cycles can not be carried too far, for in the recovery of nerve, the second part of the cycle usually includes a supernormal period, for which no analogy has been found in the recovery cycle of motoneurons. It is true that in the recovery of peripheral nerve, actual supernormality may easily be suppressed by rhythmic stimulation, but there remains a peak of excitability which disappears only after severe tetanization. In the recovery cycle of ocular motoneurons, however, the presence of even a depressed peak has not been demonstrated convincingly. Nevertheless, there seems to be no objection against using the term "subnormal phase" for the second period of the recovery cycle of motoneurons.

During activity of the nervous system, the subnormal phase, as it appears in the motoneurons, is even more apt to lead to inhibition than is anticipated from the subnormal phase in nerve, because the lack of supernormality prevents the appearance of a period of facilitation preceding inhibition. Thus the results reported in this paper lend strong support to Gasser's theory of central inhibition (1935b, 1937a, b) through the creation of subnormality. In fact, subnormality appears to be a most effective inhibitory process, for if an internuncial cell is made to fire two impulses in quick succession, its threshold must rise to a high level and remain so for a considerable period of time, with the result that further impulses delivered to the cell will be blocked.

SUMMARY

1. The recovery cycle of the synaptic excitability of the ocular motoneurons after one response consists of a protracted period of depressed excitability lasting at least 30 msec.

2. The recovery cycle of the electrical excitability of the soma of the motoneuron is like that of the axon, and may include a supernormal phase.

3. Tetanic activity does not change the early part of the recovery cycle of synaptic excitability, up to 1.5 or 2 msec., but it greatly delays recovery beyond this point. Two responses in rapid succession are sufficient to produce severe and long-lasting subnormality.

4. The significance of subnormality for the physiology of the central

nervous system is discussed and it is concluded, in agreement with Gasser, that the summation of subnormality may be one of the elementary processes underlying central inhibition.

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THE ACTION OF PITUITARY EXTRACTS ON THE COLON OF THE CAT

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In reviewing the extensive literature on the effects of pituitrin, pitressin, and pitocin on the muscular tone of the intestines, one is most impressed by the conflicting results obtained by different investigators. In man, following injections of pitressin or pituitrin, there is a general tendency toward contraction of the colon which often results in defecation (1, 2, 4, 6, 8); in the dog, cat and rabbit, however, many of the cases reported show a decrease in colonic tonus (1, 3, 7, 11, 12) following the administration of either pitressin or pituitrin (5, 9, 10).

It is probable that many of the discrepancies, as pointed out by Melville and Stehle (9, 10), are due in part to differences in the experimental technic employed, such as the use of isolated segments of colon, different anesthetic agents, or colonic fistulas. Not infrequently investigators have also failed to indicate the dose of the drug used per kilogram of body weight, and when the doses were specified, they showed wide variations. In one case, for example, a loss of colonic tonus was reported in a 14 kgm. dog given an injection of 2 pressor units of pitressin, whereas a second worker reported contraction of the colon of a 7.8 kgm. dog given an extract containing 14 pressor units.

It is the purpose of this paper to report data concerning the effects of pitressin and pitocin on the colon of the cat. The colon previously had been made opaque to roentgen rays by a technic recently reported by two of us (13). In addition, data will be presented on the approximate amount of pitressin necessary to cause colonic contraction and defecation.

METHOD AND PROCEDURE. The technic consisted in making the colon opaque to roentgen rays by injecting thorium dioxide (thorotrast) at various sites along the colon just underneath the serous membrane. After three or four weeks, the material became uniformly distributed along the entire length of the colon so as to give a clear outline of the organ with the roentgen rays.

This procedure affords a technic for investigating changes in the colon of animals that are apparently in good health. Three of the four cats

used in this investigation were given injections approximately nine months prior to study; the fourth animal was prepared about eighteen months in advance. The cats appeared normal in respect to appetite and activity. Roentgenograms were taken with the cat firmly but comfortably secured in a specially constructed holder which restricted body movement without producing any evident emotional disturbances. After a control picture had been taken, the animal, without being removed from the holder, was given an intravenous injection of either pitressin or pitocin[1] (0.15 cc. per kgm. of body weight). Following this, roentgenograms were taken at intervals of two to five minutes for thirty-five minutes. To avoid distortion of the colon from a distended stomach all experiments were carried out fifteen to eighteen hours after the last feeding.

With these successive roentgenograms it was possible to measure and compare any change in the size or shape of the organ before and after injection of the drug. Measurements of the colon were made by tracing

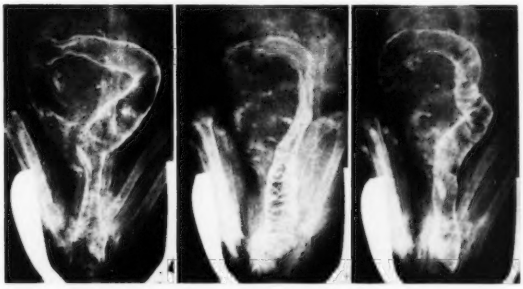

Fig. 1. *Left*, normal size of cat's colon (control); *middle*, stage of greatest contraction following an injection of pitressin, and *right*, showing recovery after effect of pitressin has disappeared.

its outline on graph paper and computing its area in square centimeters. While this method permits relatively accurate determination of the area of the colon as pictured in the roentgenogram, it must be admitted that actual changes in volume cannot be estimated because of possible distortions of the colon within the abdominal cavity.

Results. The data obtained showed that the effects of pitressin were not the same in the four cats studied. With a dose of 0.15 cc. per kgm. of body weight (3 pressor units) two of the animals consistently showed marked colonic contraction; this reached its maximum within fifteen minutes and was then followed by a general relaxation and return to normal. In some cases defecation followed injection, leaving the colon definitely smaller than at the beginning of the experiment. These positive effects of pitressin on the colon of the cat are shown in figure 1. The other two

[1] Parke Davis.

cats did not show evidence of colonic contraction. On some occasions a tendency toward actual relaxation or loss of tonus occurred. In neither case, however, did the colonic area increase more than 10 per cent over that of the control. This difference was in distinct contrast to the previous two cats whose colons showed a decrease in area of 40 to 50 per cent following injections of pitressin. When pitocin was injected in similar

TABLE 1

Effect of various doses of pitressin on defecation

CAT	WEIGHT	EXPERIMENT	CUBIC CENTIMETER OF PITRESSIN PER KILOGRAM BODY WEIGHT		
			0.1	0.15	0.2
	kgm.				
1	2.9	1	Negative*		
		2	Negative		
		3		Positive	
		4			Positive
2	2.8	1	Negative		
		2	Positive		
		3		Positive	
		4			Positive
3	3.2	1		Negative	
		2			Positive
		3			Positive
		4			Positive
4	3.1	1		Negative	
		2			Positive
		3			Positive
		4			Negative

* If defecation occurred within fifteen minutes the test was positive, if not it was considered negative.

doses no response was noted in any of the four cats. Likewise in a few experiments with pituitrin no response was recorded.

So consistent a difference in response to injections of pitressin suggested a possible difference in threshold response. To test this hypothesis, a number of experiments were performed on these four cats using varying quantities of pitressin per kilogram of body weight. In this study defecation was taken as the criterion of colonic activity since it has been shown usually to accompany colonic contraction. Instead of being kept in the animal holder, the cats were observed in their cages following injections of pitressin. Injections were made intravenously in the following amounts: 0.1 cc., 0.15 cc. and 0.2 cc. per kgm. of body weight. Each cat was given injections on four different occasions with varying doses

(table 1). If defecation occurred within fifteen minutes the test was referred to as "positive."

Cats 1 and 2 which had previously shown only colonic contraction or contraction and defecation when given 0.15 cc. of pitressin defecated on only one occasion when the dose of pitressin was reduced to 0.1 cc. per kgm. Apparently this was below the threshold level. The other two cats (3 and 4) as in the previous experiment failed to defecate following the injection of 0.15 cc. of pitressin, but did so in five of the six experiments following the injection of 0.2 cc. This, we feel, may be taken to indicate that their threshold response was higher than that of the other two cats.

It is our opinion, therefore, that this contraction of the colon following injections of pitressin is a response secondary to some other physiologic disturbance. Throughout these experiments it was observed that when the dose of pitressin was large enough to induce colonic contraction or contraction and defecation, the animals showed sudden and marked symptoms of a physiologic disturbance involving the respiratory and circulatory systems and resulting as well in dilated pupils and profuse salivation.

SUMMARY

Studies on cats whose colons had previously been made opaque to roentgen rays with thorium dioxide showed that there is a threshold in the dosage of pitressin which must be reached before colonic contraction occurs. It has been shown that 0.1 cc. of pitressin per kgm. of body weight appears to be the subthreshold level, whereas 0.2 cc. was sufficient to cause defecation in a large number of the experiments.

The observations suggested that contraction of the cat's colon subsequent to injections of pitressin was possibly secondary to some other major physiologic disturbance.

There was no colonic response to equal doses of pitocin or pituitrin.

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OBSERVATIONS ON THE MODE OF ACTION OF PIPERIDINOMETHYLBENZODIOXANE (933F)

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The synthetic dioxane derivative 933F has been found to reduce or abolish the responses of smooth muscle to adrenine while leaving relatively unimpeded the effects of nerve stimuli (DeVleeschower, 1934). Among the hypotheses put forward to account for this difference is that of Rosenblueth and Cannon (1936) who suggested that 933F polarizes the effector cells, thus limiting the penetration of circulating chemicals without affecting the activity of mediators liberated intracellularly by the nerves. An important corollary to this hypothesis is that, in organs where the sympathetic nerves end outside the cells, 933F should reduce equally the effects of nerve stimulation and of adrenine. There is some evidence that in the submaxillary gland the nerve endings are extracellular (Stöhr, 1928); it was therefore suggested by Doctor Cannon that we undertake a study of the effects of 933F on this organ. As the work progressed other possibilities arose which were included in the study.

METHOD. Cats anesthetized with dial (Ciba, 0.7 cc. per kgm. intraperitoneally) were used. The submaxillary duct and chorda tympani were exposed on one side through an incision on the under side of the jaw. A fine glass cannula was tied into the duct and connected by rubber tubing to a capillary tube calibrated in hundredths of a cubic centimeter. The flow of saliva as it passed each division was observed and was recorded by means of signal-magnet writing on a kymograph. In a few experiments the number of drops falling from a calibrated capillary (1 drop = 0.02 cc.) were registered in a similar manner. Blood pressure was recorded with a mercury manometer connected with a femoral artery or with the carotid contralateral to the gland studied. Contractions of the nictitating membrane were recorded isotonically on a kymograph.

The cervical sympathetic nerves were approached in the neck well away from the submaxillary gland. The stimulating current was supplied to a pair of shielded electrodes from a Harvard inductorium.

In a few experiments the adrenal glands were removed; but as they were

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found to play no rôle in the responses studied, the procedure was ordinarily omitted.

The drugs used were the following: adrenalin (Parke, Davis); cocaine hydrochloride (Merck); pilocarpine nitrate; and piperidinomethyl-3-benzodioxane (933F), the last being kindly supplied by Dr. D. Bovet. All injections were made into a femoral vein.

RESULTS. A. *Effect of 933F on salivary secretion.* In every preparation tested, 933F depressed the salivary response to adrenalin more than it impaired an equivalent secretion produced by nerve stimulation. The

TABLE 1

Salivary response (in 0.01 cc.) to sympathetic stimulation and to injected adrenalin before and after 933F

EXPERIMENT NUMBER	SYMPATHETIC STIMULATION			ADRENALIN			DOSE 933F
	Before 933F	After 933F	Per cent reduced	Before 933F	After 933F	Per cent reduced	
							mgm. per kgm.
1	7.	7.5		10 (100γ)	4	60	2
	7.5	5	30	4 (100γ)	3	25	4
	Total change		30			66	
2	11	9	18	15 (100γ)	15		2
	9	8	11	15 (100γ)	8		2
	Total change		28			44	
3	4.5	3	33	5.5 (100γ)	0	100	2
4	18	12	33	20 (60γ)	0	100	2
5	11	5.5	55	20 (100γ)	0	100	2
6*	6	4.5	25	22 (100γ)	2	90	2
7*	4	1	75	14 (100γ)	2	86	2
8†	9	5	44	48 (100γ)	2	90	2
9†	7	6.5	7	10 (100γ)	1	90	2
Average change			35			85	

* Experiments carried out after the injection of cocaine, 5 mgm. per kilogram.

† Received pilocarpine, 0.1 mgm.

effect of small doses of adrenalin proved to be relatively more affected by 933F than did large doses, a relationship which is also true for the responses of the nictitating membrane (cf. the records published by Bacq and Fredericq, 1935). In order to avoid discrepancies due to this fact, in comparing the effects of 933F on the action of adrenalin and on nerve stimulation, the response to a dose of adrenalin slightly larger than that necessary to match the response produced by a maximal 30-second tetanic stimulation applied to the nerves, was ordinarily selected for comparison. The results of all the experiments are tabulated in table 1. A typical experimental record is reproduced in figure 1. In a few experiments in

which the flow of saliva was inadequate for accurate quantification, cocaine or pilocarpine was given to increase the responses. If the latter substance was used, the secretion induced by the drug itself was allowed to subside before the test procedures were carried out. Although the results were consistent with the other experiments (see table 1), these drugs tended to complicate the interpretation of the experiments and were avoided whenever possible.

During the course of the study it became evident that the responses to adrenalin given soon after a maximal stimulation were occasionally enhanced (cf. Simeone, 1938, for a similar phenomenon in the nictitating

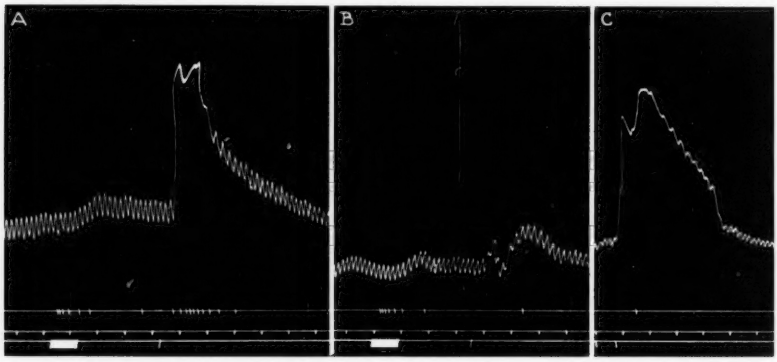

Fig. 1. Pilocarpine (100γ) injected previously. Upper record: blood pressure; first signal line: salivary secretion in 0.01 cc.; second signal line: time in 30-second intervals.

A. Before 933F. Faradic stimulation of cervical sympathetic and adrenalin, 100γ, as indicated by the third signal line.

B. After 933F (2 mgm. per kgm.). Stimulation of the cervical sympathetic and adrenalin, 100γ.

C. Adrenalin (100γ) followed by clamping the aorta at the diaphragm, in order to raise the blood pressure.

membrane). In comparing the secretion produced by adrenalin before and after 933F, only those responses were considered which occurred at equal times after sympathetic stimulation or so long afterwards that the enhancing effect of the stimulation was insignificant.

It was remotely possible that the reduction of the adrenalin response after 933F was due to the fact that the rise in blood pressure ordinarily produced by adrenalin was nearly abolished by the drug. This possibility is unlikely since the local vasoconstrictor action of the drug would also be abolished so that little change in blood flow would be expected. Experiments in which the blood pressure was artificially raised by clamping the

aorta at the diaphragm (after an adrenalin injection) failed in fact to restore the response (fig. 1C).

Since the foregoing observations showed that 933F preferentially depressed the responses to adrenalin over those of nerve stimulation, even in organs where the nerves appear to end outside the cells, they failed to support the hypothesis that the drug acts by decreasing cellular permeability. Further experiments therefore suggested themselves.

B. *Effect of 933F on adrenalin responses already in progress.* Bacq (1936a and b) has shown that 933F abolishes the prolonged contractions of the nictitating membrane caused by ephedrine, or by adrenalin when its rapid destruction has been prevented by pyrogallol. Experiments such as that illustrated in figure 2A demonstrate that this is also true for the shorter responses of the nictitating membrane or blood pressure to

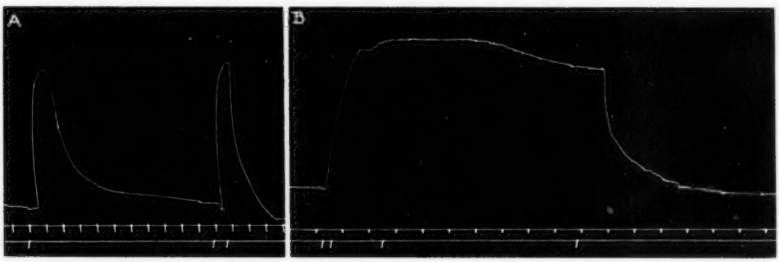

Fig. 2. Dial anesthesia. Upper record: contractions of the nictitating membrane, after section of the cervical sympathetic.

A. First signal: adrenalin, 100γ. Second signal: same. Third signal: 933F injected (2 mgm. per kgm.).

B. Same preparation after effect of 933F had worn off. First signal: adrenalin, 100γ. Between second and third signal cocaine (5 mgm. per kgm.). Fourth signal: 933F (2 mgm. per kgm.).

adrenalin alone. A similar but more striking depressing action of 933F can be demonstrated on an adrenalin response prolonged by cocaine (fig. 2B). In these cases the drug was given after the height of the adrenalin response was reached, when most of it had left the blood stream and very probably gained access to the smooth muscle cells. That the result was not due merely to a nonspecific relaxing action of 933F on smooth muscle was shown by experiments in which the blood-pressure rise or membrane contraction was produced by ergotoxine. A subsequent injection of 933F did not significantly affect them.

C. *Effect of 933F on adrenine destruction in vitro.* Bacq and Bovet (1935) stated that 933F slightly accelerated the destruction of adrenalin *in vitro*. Since most, if not all, of the phenomena attributed to the drug could be explained if it appreciably increased the rate of destruction it seemed

desirable to have more quantitative evidence on this point. The following experiments were therefore carried out by one of us (K. L.).

A solution was made containing 1 part of adrenalin to 100,000 parts of bicarbonate Ringer's solution, at pH 8.03. To a portion of this solution 933F was added to obtain a concentration of 1:10,000. Both solutions were then exposed to a constant stream of air for periods up to 48 hours. From time to time tests of adrenalin activity in these solutions were carried out as follows. Various dilutions of the test solution were made and assayed on the hypodynamic frog-heart preparation (Straub). The adrenalin activity of the test solution was then plotted as the greatest dilution which showed a definitely positive effect, against the time allowed

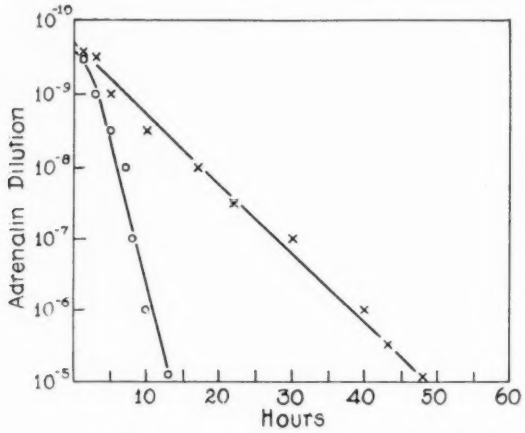

Fig. 3. Curves showing relative rates of inactivation of adrenalin without (crosses) and with (circles) 933F (1:10,000), in oxygenated alkaline Ringer's solution. Ordinates: greatest dilution of the solutions which showed a positive inotropic effect on the isolated frog heart (Straub). Abscissae: time elapsed after original mixing of the solutions.

for destruction. The hearts were checked frequently during the experiments with fresh dilutions of adrenalin to insure that their sensitivity had not changed. Three such experiments were made, all of which showed that inactivation proceeded about three times more rapidly in the solutions containing 933F than in the solutions containing adrenalin only. A typical experiment is illustrated in figure 3.[2]

Objection might be raised that the 933F present in the test solution prevented the typical adrenalin effect (Shen, 1937). With the method

[2] Since this work was done Bayer and Wense (1938) have shown that 933F accelerates the destruction of adrenalin by acetaldehyde *in vitro*.

employed, however, the concentration of the drug reached significant proportions (i.e., 1:10,000) only when the adrenalin activity had been reduced to a minimum. In other words, only the last point on the curve shown in figure 3 could have been importantly affected. There is reason to believe, moreover, that even this concentration of 933F, although it may depress the heart after relatively prolonged or repeated perfusion, does not significantly inhibit the stimulant effect of adrenalin except in rather special circumstances (cf. Hill and Myers, 1938). Under the experimental conditions employed in the present tests, no important interference of 933F with the usual inotropic effect of adrenalin could be made out.

Discussion. The experiments reported in section A show that the effect of 933F on the responses of the submaxillary gland is essentially the same as in other organs. It prevents the effects of adrenalin without comparably impeding nervous excitation. If the evidence that the nerves in the salivary glands end outside the cells could be accepted unreservedly, these results would by themselves be seriously at odds with the hypothesis that 933F exerts its effect by rendering cells impermeable to chemical excitants. Unfortunately, no certainly exists as to the relation of the nerve endings to the secretory elements (Boeke, 1932). The experiments outlined in section B of this paper, however, confirm the impression that 933F does not act by rendering the effector cells impermeable, since it was found that the effects of adrenalin were abolished by the drug when administered at a time when it could be safely assumed that adrenalin had reached and activated the muscle cells.

One other suggestion has been made to account for the relative lack of effect of 933F on the responses to sympathetic stimuli. Monnier and Bacq (1935) have proposed that the nerves not only liberate an adrenine-like chemical mediator, but are also capable of activating directly the effector cells by means of their action potentials. However well this explanation may be thought to apply to some of the phenomena in the nictitating membrane (cf. Rosenblueth and Cannon, 1936, for a full discussion) which possesses the requisite initial potential, it is difficult to apply to the present situation. No such initial action potentials have been demonstrated in the submaxillary gland (cf. Rosenblueth, Forbes and Lambert, 1933), and the same difficulties arise here as confronted Ross (1937) when he tried to apply the dual transmission theory to the uterus of the cat.

Since the only direct evidence available to explain the mode of action of 933F is the fact that it accelerates the inactivation of adrenalin *in vitro* (section C), it is pertinent to inquire whether it is possible to account for all the observed phenomena in the animal on this basis. The destruction of adrenalin is much more rapid in living tissues than in Ringer's solution

or in blood *in vitro* (Bain, Gaunt and Suffolk, 1937). It is not probable that 933F could increase the slow rate of destruction by blood sufficiently to account for its check on adrenalin activity; and, in fact, a few experiments in which adrenalin and 933F were held for short periods in occluded veins before being allowed into the general circulation confirmed this impression. It is more probable, however, that doubling or tripling the ordinarily rapid rate of adrenalin destruction in the tissues would alter the maximal concentration possible at a given time after its injection. The injected adrenalin would reach the tissue relatively slowly via the circulation, and destruction of the first portion would be well under way by the time the last of it arrived. Indeed, such as assumption is implicit in the explanations offered for the sensitizing action of procedures which are thought to slow adrenalin destruction—denervation (Simeone, 1938) and the injection of antioxygens (Bacq, 1936a). Conversely, it follows that anything which accelerates destruction in the tissues results in smaller responses. Depression of a variety of excitatory responses to adrenalin by 933F has, indeed, been reported (see Shen, 1937, for references). Bovet and Simon (1936) have found that it also impairs the inhibitory responses of the intestine, and although Bacq and Bovet (1935) report an instance in which the inhibition of the cat's uterus was increased by 933F, Ross (1937) found depression of inhibition in all his experiments.

It is difficult to obtain information of quantitative significance from observations on the blood pressure; probably, however, a rapid rate of adrenalin destruction could convert an apparently large dose into a small concentration at the effector cells and thus give rise to the well-known vasodilatation produced by small doses. Such an explanation could account for the so-called reversals of the blood pressure responses reported for 933F (DeVleeschower, 1934).

The relative lack of effect of the drug on nerve stimulation is more difficult to deal with. It is possible, however, that a mediator liberated close to or inside the effector cells would be exposed to destruction for a far shorter time preceding contraction than would adrenalin arriving via the blood stream.

Without more quantitative information than at present exists, further speculation cannot be fruitful, but it may be worth while to point out that the sympathetic mediator may not always be adrenine, although excellent evidence points to their similarity (cf. Cannon and Rosenblueth, 1937). Differential effects of drugs on nerve stimuli and injected adrenalin might then be made on the basis of the different excitants involved in the two types of stimulation. Evidence has been accumulating to support this view. Not only is the substance given off to the blood after sympathetic stimulation in several respects different from adrenine (Cannon and Rosenblueth, *loc. cit*); but Kennard (1937) and Ross (1937) have had to

make rather elaborate subassumptions in order to reconcile their observations with the view that the mediator is identical with adrenine. Even more striking are the experiments of Acheson (1938), who found that after appropriate doses of ergotoxine, adrenalin causes a pure relaxation of the nictitating membrane, while nerve stimulation produces an equally pure rise of about the same extent. These results, though explicable on the basis of the non-identity of the sympathetic transmitter with adrenine, serve principally to emphasize the difficulty of including all the known phenomena in a single, thoroughly consistent theory.

SUMMARY

1. The dioxane derivative 933F markedly reduces the responses of the salivary gland to injected adrenalin. In spite of the probability that the nerves end outside the effector cells, the responses to sympathetic nerve stimuli are less seriously impaired (fig. 1, table 1).

2. After the nictitating membrane or the blood pressure has responded fully to adrenalin, the injection of 933F rapidly curtails the response (fig. 2).

3. The destruction of adrenalin *in vitro* is significantly accelerated by 933F (p. 408).

4. Observations 1 and 2 are shown to make it difficult to explain the action of 933F by changes of cellular permeability.

5. Some other plausible explanations of the action of 933F are discussed.

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CHANGES IN COMPOSITION OF SWEAT DURING ACCLIMATIZATION TO HEAT

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In animals that undergo large fluctuations in rate of heat production there is usually a provision for temperature regulation that depends for its effectiveness on the high heat of vaporization and the high specific heat of water. The evaporation of water from the lungs, respiratory passages, and tongue by hyperventilation provides small, warm-blooded animals with an adequate means for cooling. Even the poikilothermous lizards may pant if their body temperatures rise above the zone of indefinite survival. Many large mammals do not pant but depend on the secretion and evaporation of sweat on the surface of the body for dissipation of heat.

Either procedure is disturbing to the internal economy; aside from the loss of water there is, in panting, a reversible change in acid-base balance that arises from excessive elimination of CO_2 (1) and, in sweating, a depletion of electrolytes from body fluids (2). Our earlier studies of salt economy (3, 4) have suggested that the magnitude of the latter disturbance is related to degree of acclimatization, individual characteristics, and the level of activity of the sweat glands. During the past year these dependent relations have been measured quantitatively, the subjects being members of the Harvard Desert Expedition.[2]

METHODS. Before starting each period of observation, the subject took a shower bath, was weighed to the nearest decigram, and donned light clothes. Ordinarily he then walked over a standard course at a uniform rate. In some instances, the rate was varied and on other days some of us played tennis or in a few experiments sat in the sun.

In addition to the measurements described by Adolph and Dill (5),

[1] Died December 14, 1937.

[2] The members of the party were, besides the authors, Dr. E. F. Adolph of the University of Rochester, Dr. R. A. McFarland, Frank Consolazio, and David B. Dill, Jr. We are indebted to Mr. John C. Page, Commissioner of the U. S. Bureau of Reclamation, for laboratory space in the Municipal Building at Boulder City, and to Mr. Ralph Lowry, Resident Engineer, and Mr. Ely, City Manager, for their coöperation.

the rates of pulmonary ventilation and energy expenditure were determined in one or more experiments, using the Douglas bag technique. A thermometer placed near the insulated expiratory valve gave the temperature of expired air. The loss of weight during the experiment, corrected for *a*, water intake; *b*, insensible loss from the skin (400 cc. per day); *c*, water from the lungs and respiratory passages (calculated from the volume, composition, and temperature of inspired and of expired air), and *d*, the difference between the weight of CO_2 expired and of oxygen used, corresponded to the volume of sweat excreted in the period of observation. The free movement and low humidity of desert air effected complete evaporation of sweat in situ. Non-volatile constituents left on the skin were collected in a bathtub by washing the subject with 5 liters of distilled water; the clothes were washed in the same water before it was sampled for analysis. Sodium was determined by the method of Butler and Tuthill (6), potassium by the method of Shohl and Bennett (7), chloride by the Volhard procedure, and nitrogen by Kjeldahl. In applying the first two methods, suitable aliquots, treated with H_2SO_4, were evaporated to dryness and ashed below visible red heat. Sodium, potassium, and total nitrogen were determined in samples returned to Boston in sealed ampoules. In many cases sweat was collected directly by wearing a rubber glove held snugly about the wrist with a rubber band. In such samples pH by glass electrode and lactic acid by the usual procedure (8) were determined.

Results. The relation of the concentration of chloride in sweat to the rate of sweating is shown in figure 1. The periods of observation covered by these measurements range from 5 days in the case of J to 47 days in the case of C. The periods required for acclimatization, to be defined arbitrarily below, ranged from 1 day for H to 10 days for C. Measurements made during this transition state were not used in figure 1, except in the case of J. There is no reasonable doubt that sweat tends to become more concentrated as it becomes more profuse. In other words, the more active the sweat glands, the less effectively is the chloride ion held back.

One other fact is established by figure 1; there are unmistakable differences between individuals in respect to the chloride concentration in sweat. Subjects D and J have the most concentrated sweat; A and H stand at the other extreme. There were only 4 observations on D below 30 mE (milli-equivalents per liter) and none as high as 30 mE on A or H. No basis for these idiosyncrasies was revealed by analysis of blood, to be reported elsewhere.

While the trends indicated by the lines drawn in figure 1, derived by the method of least squares, are almost certainly significant, the deviation of the points from the lines indicates that the rate of sweating is not the

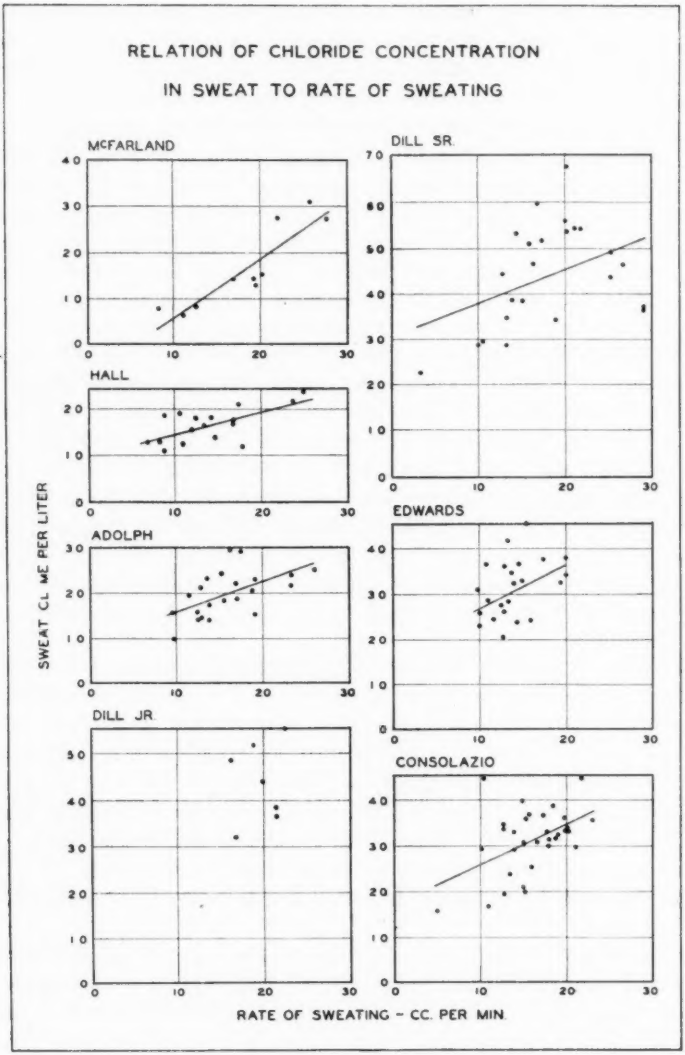

Fig. 1

only factor concerned. The concentration of sweat may depend on rate of heat production, external temperature, intensity of sunlight, and the nature of the clothes worn, but we could not tell from studies of these

factors whether they modify the product of sweat gland activity otherwise than indirectly through influence on the rate of sweating.

During the winter of 1936–37 experiments were carried out on 6 of the men who were to make up the Boulder City party. Desert conditions were approximated by heating a room to about 43°C. and keeping the relative humidity at about 10 per cent. Enough air movement was provided to evaporate sweat in situ. Walking on a moderate grade on a treadmill in this room raised the metabolic rate to about 5 times the

TABLE 1

*Excess concentration of sweat chloride over the value characteristic of the acclimatized state**

(The rate is expressed in cubic centimeters per minute and chloride concentration in mE per liter of sweat)

DATE	A		C		D		E		H		M	
	Rate	Δ Cl	Rate	Δ Cl	Rate	Δ Cl	Rate	Δ Cl	Rate	Δ Cl	Rate	Δ Cl
Boston:												
Winter of 1936–37	3.6	54.6	14.0	33.7	12.3	52.8	11.2	35.6	9.8	12.1	13.0	37.5
Boulder City:												
First day	10.3	17.5	11.8	18.1	12.6	31.6	13.1	29.8	12.9	4.5	21.3	10.9
Second day	6.6	14.8	12.0	5.4	9.3	25.2	12.2	8.9			20.4	2.6
Third day	10.1	10.5	16.5	3.0	11.7	21.6					18.4	4.5
Fourth day	9.2	12.3	18.0	17.1	10.5	9.9						
Fifth day	10.3	5.9	14.0	6.5	11.4	8.2						
Sixth day	15.6	4.3	12.5	8.2	11.8	20.8						
Seventh day			13.0	12.6								
Eighth day			16.5	23.1								
Ninth day			17.2	20.4								
Tenth day			16.7	15.2								

* The concentration of chloride in sweat may be calculated for each case by adding ΔCl, given in the table, to the sweat chloride in the acclimatized state, indicated by the curves in figure 1.

basal level and, with the exception of one subject, produced a rate of sweating comparable to that observed later on a moderate day at Boulder City. The average concentration of chloride in sweat was about twice as great in Boston as was later observed at Boulder City when the rate of sweating was the same. Table 1 shows the excess of chloride over that characteristic of the acclimatized state. After arriving at Boulder City, from 1 to 10 days elapsed before sweat chloride reached, or fell below, the trend of subsequent observations. This period of transition or of acclimatization was shortest in the case of H, who reached Boulder City after a 3-day trip from Durham, North Carolina, where he had

already experienced much hot wheather. It was longest in the case of C, who after 10 days doubled the quantity of strenuous exercise he had been doing. Possibly the completeness of acclimatization depends both on the duration and on the intensity of activity of the sweat glands.

Observations on sweat of the post-acclimatization period have been segregated into two numerically equal groups for each subject. One group includes the experiments with lowest rates of sweating, and the other, experiments with the highest. The mean values, collected in

TABLE 2

Mean values for sweat constituents

	NUMBER OF OBSERVATIONS	RATE	N	Cl	Na	K
A. Low rates of sweating						
		cc. per min.	*mgm. per. l.*	*mE per l.*	*mE per l.*	*mE per l.*
A	11	12.4	282	17.6	16.1	2.4
C	15	12.9	351	26.7	26.7	3.2
D	12	13.0	304	39.1	40.4	3.0
E	8	12.6	289	29.3	24.2	3.3
H	8	9.8	513	14.8	7.3	2.9
J	3	17.2	330	43.8	38.6	1.9
M	5	13.6	256	10.1	9.9	2.3
Mean		13.1	332	25.9	23.3	2.7
B. High rates of sweating						
A	11	19.2	251	22.8	23.7	3.2
C	15	19.3	296	33.8	33.5	2.9
D	12	23.0	236	48.5	52.7	3.1
E	8	17.2	259	34.0	33.7	4.2
H	8	18.3	403	17.8	13.3	3.0
J	3	21.8	306	43.5	42.9	2.3
M	5	23.1	212	23.0	23.3	2.8
Mean		20.3	280	31.9	31.9	3.1

table 2, show that while chloride, sodium, and potassium become more concentrated as sweat becomes more profuse, the total nitrogen content decreases. There appears to be no doubt about the reality of the latter observation, for it is true for each subject. Individual differences, revealed in respect to chloride in figure 1, are equally evident in respect to other constituents. If one arranges the subjects in the order of increasing concentration of nitrogen, 5 of the 7 are in the same order in the two sets of observations. The same is true of potassium.

The three ions determined in every experiment, sodium, potassium,

and chloride, show an approximate acid-base balance between them, indicating that a balance exists between undetermined anions and cations, chiefly calcium, ammonium, and lactate. The conditions of sweat collection were not suited for measuring precisely acid-base balance since the pH may be altered by evaporation. In a number of experiments we attempted to evaluate the lactate ion concentration in body sweat by determining the total lactic acid in washings and the pH of hand sweat collected simultaneously,[3] employing the titration curve for lactic acid given by Fishberg and Bierman (9) for calculating the proportion of lactic acid present as lactate. Table 3 summarizes the results of such experiments. The sum of undetermined cations, chiefly ammonium and calcium,[4] appears to be low in the sweat of A and D, moderate in C and M, and relatively high in H. It is significant in this connection that the sweat of H was highest in total nitrogen content.

TABLE 3

Acid-base balance

	NUMBER OF OBSERVATIONS	Na	K	Cl	TOTAL LACTIC ACID	pH	(LACTATE)	Σ ANIONS − Σ CATIONS
		mE per l.	*mE per l.*	*mE per l.*	*mM per l.*		*mE per l.*	*mE per l.*
A	1	27.1	2.9	24.9	5.7	4.82	5.0	+0.1
C	2	31.4	3.2	32.4	5.7	5.11	5.2	+3.0
D	5	50.4	3.4	46.5	6.8	6.09	6.8	−0.5
H	3	13.7	2.9	17.4	10.9	4.50	8.7	+9.5
M	1	10.8	2.7	12.8	6.9	4.76	5.9	+5.2

It has been suggested by Whitehouse (13) that sweat is normally alkaline (pH 7.2 to 7.8); he believes that acid sweat is obtained only when the skin is not carefully cleaned. We cannot agree with this suggestion. On one occasion, after washing their hands thoroughly, C and D each donned a rubber glove and collected four successive samples in a 2-hour walk. The pH values on C were 5.64, 5.45, 5.39 and 5.20, and those on D were 6.41, 6.66, 6.92 and 7.50. While the sweat became more alkaline in one man, it became more acid in the other. Neither do we agree with the suggestion of Fishberg and Bierman (9) that sweat is acid enough to permit considerable base-saving by secretion of lactic acid. Their conclusion may apply to the non-acclimatized subject, but it does not apply to the man who has become adapted to dry heat.

[3] This is not unobjectionable since the pH of hand sweat may differ from that of sweat excreted on other body surfaces.

[4] According to Talbert, Finkle and Katsuki (10) and Mosher (11), the NH_4^+ content of sweat may range from 4 to 14 mE per liter. Calcium has a concentration of about 2-3 mE (12).

In most cases chloride concentrations in body sweat and hand sweat (table 4) were of the same order of magnitude, but this was not true of lactic acid; its concentration may be two or three times as high in sweat collected in a glove as in sweat produced over the entire body surface. We are not sure whether the latter difference is inherent or whether it may depend on the method of collection. The skin temperature when a rubber glove is worn is several degrees above its usual temperature, and the function of the sweat glands may be modified thereby. Whitehouse looks upon lactic acid in sweat as a product of sweat gland activity, but it hardly seems likely that the metabolism of sweat glands should be anaerobic at a time when the skin is so well supplied with blood.

TABLE 4

Chloride in body sweat and hand sweat

SUBJECT	NUMBER OF OBSERVATIONS	CHLORIDE		
		Body	Hand	Δ
		mE per l.	*mE per l.*	*mE per l.*
A	2	20	22	+2
C	6	29	38	+9
D	12	41	41	0
H	5	17	19	+2
J	3	51	67	+16
M	1	13	13	0
Mean		28	33	+5

Discussion. An increase in concentration of chloride in sweat with increased rate of sweating has been reported by Kittsteiner (14) and Viale (15), but could not be detected by Talbert and Haugen (16). The lack of agreement may depend on the failure to appreciate the importance of acclimatization and of individual differences. When these are taken into account, there is no reasonable doubt that chloride concentration is higher the more profuse the rate of sweating. However, it must be admitted that there are variations in composition of sweat even in the same individual for which no explanation is at hand.

The adaptive process whereby man becomes inured to high temperatures clearly consists, in part, of the decreasing salt content of sweat. After acclimatization man will lose less salt, and the satisfaction of his thirst will keep the volume and concentration of his body fluids more nearly optimal. Possibly heat exhaustion and certainly heat cramps are in consequence less apt to occur. It is equally clear that some subjects remain susceptible to heat cramps because their sweat glands do not hold back salt effectively. Even after several weeks at Boulder City, D put

out the equivalent of his daily salt intake in 3 hours of strenuous exercise on a hot day.

Sweat in man acclimatized to high temperature must be looked upon as a secretion in which one constituent, K^+, has about the same concentration as in interstitial fluid. Na^+ and Cl^- may be one-tenth as concentrated as in interstitial fluid, while H^+, NH_4^+, and the lactate ion may be raised to much higher concentrations than are observed in the body. In man adapted to life in the hot desert lactic acid excretion is kept at a low level; it may be balanced in part by excretion of ammonia and calcium.

SUMMARY AND CONCLUSIONS

The properties of sweat depend on acclimatization, the rate of sweating, inherent characteristics of the individual, and probably other factors. It becomes more dilute with adaptation to hot atmospheres. Its inorganic constituents increase in concentration as sweating becomes more profuse; at the same time, nitrogen excretion diminishes. The susceptibility of some individuals to heat cramps no doubt depends in part on the inability of their sweat glands to reduce sodium chloride loss to a low level.

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OXYGEN CONSUMPTION OF WHITE CELLS FROM PERITONEAL EXUDATES[1]

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Where a peritoneal exudate is produced in the rabbit by the method of Mudd, Lucké, McCutcheon, and Strumia (1929), it can be shown that the number of white cells appearing in the exudate is greater than the number in the circulating blood, and that this withdrawal of cells is followed by a stimulation of the bone marrow (Ponder and MacLeod, 1938). If exudates are produced in rapid succession, the cells appearing in the circulation and in the exudates become younger and younger; it is with the O_2 consumption of these increasingly immature cells that this paper is concerned.

METHODS. 1. *Production of exudates.* About 300 cc. of sterile 1 per cent NaCl, or of Ringer's solution,[2] are injected intraperitoneally; 18 hours afterwards the fluid is drawn off without further injection of saline, and is received into buffered Ringer's solution (pH 7.35) with only a few milligrams of citrate added to prevent coagulation. The exudate is transferred to centrifuge tubes and is spun at the rate of about 1000 r.p.m. for 2 minutes; the supernatant fluid is then replaced by sufficient Ringer's solution to make a suspension containing from 40,000 to 60,000 cells per mm.[3] The exact number is found by making a count in the usual way.

When a single exudate is produced, at least 95 per cent of the cells are polymorphs. Examination of the cells of the blood stream, from which the cells of the exudate are necessarily derived, shows that the prevailing type of polymorph is the cell of class I of the polynuclear count, i.e., a young cell just liberated by the stimulated marrow. If exudates are produced repeatedly at 2 day intervals, the prevailing type of granulocyte in the blood stream becomes successively the very young neutrophile, the metamyelocyte, and finally the myelocyte. While we have not been

[1] The expenses of this investigation were defrayed by a grant from the Ella Sachs Plotz Foundation.

[2] The composition of the Ringer's solution we have used is: NaCl, 43.75 grams, KCl, 1.1 gram, $CaCl_2$, 1.18 grams, 280 cc. M/15 KH_2PO_4, 720 cc. M/15 Na_2HPO_4, and water to make up to 5 litres.

successful in enumerating the different types of cell present in the exudates (supravital staining being not altogether satisfactory) there is no doubt but that the cells are increasingly immature, and correspond closely to the prevailing type in the circulation.

2. *Respirometry.* The measurements of O_2 consumption were made in Fenn respirometers in a water bath at 37.5°C., controlled to within 0.01°C., and rocked to and fro through an arc of 20° at the rate of 120 per minute. Two cubic centimeters of the cell suspension are placed in one of the cups of the respirometer and in the other cup is placed the same volume of Ringer. The respirometer is equilibrated in the water bath for 20 minutes, at the end of which time the O_2 consumption is measured at 10 minute intervals for one hour. We have found that these cells breathe at a steady rate for at least three hours.

In previous experiments on the metabolism of leucocytes (Ponder and MacLeod, 1936) the respiration was expressed in terms of Q_{O_2}, and considerable variation in this figure for normal cells was found. This we since have found to have been due to small errors in estimating dry weights, etc. On recalculating these figures in terms of O_2 consumed per million cells, we have found a much closer agreement between the respiration of cells of individual rabbits, and the average O_2 consumption of cells from 40 normal rabbits turns out to be 0.40 mm^3 of O_2 consumed per million cells per hour, with a coefficient of variation of only ± 1.5 per cent. Comparing this figure with Barron and Harrop's (1929) figures, we find it to be less than half that found by them; a probable reason for this is that the cells used by Barron and Harrop were immature cells from leukemic blood.

Results. *a.* Table 1 shows the O_2 consumption in mm^3 $O_2/10^6$ cells/hr. of the cells recovered from four successive exudates taken at intervals of 10 days from six adult rabbits. The average O_2 consumption of the cells of the first exudates was somewhat lower than the normal figure (0.40 mm^3 $O_2/10^6$ cells/hr.), but the figures for each animal agree closely. The cells of the second exudate, however, show a marked increase in respiration, and those of the fourth exudate show a 50 per cent increase in respiration over the original rate.

It is difficult to relate this increased respiration with the morphology of the cells, as supravital or fixed smear studies of the cells of the exudate give only a rough idea of cell structure and general idea of age. There is no doubt, however, that the cells of each successive exudate are increasingly youthful. Previous attempts to relate the youthfulness of white cells to their metabolism have been contradictory; thus Daland and Isaacs (1927) found that immature white cells have a lower O_2 consumption, but Barron and Harrop (1929) found no appreciable difference between the metabolic activity of mature and immature leucocytes. These

experiments, however, were not done on pure white cell suspensions, but on samples of whole blood from cases of leukemia, etc.

b. In order to substantiate this relation between the age of the cell and its metabolism, rabbits were injected with 2 mgm. of sodium nucleinate per kilo. This produced a marked deflection of the polynuclear count, so much so that the cells in the fixed smear were nearly all metamyelocytes. A striking characteristic of these cells is their intense basophilic granulation, which in nearly all cases was sufficient to obscure the nucleus. These cells are similar to those which appear in the blood stream after repeated exudates, but the basophilic granules are larger, more numerous, and more

TABLE 1

EXUDATE	ANIMAL 1	ANIMAL 2	ANIMAL 3	ANIMAL 4	ANIMAL 5	ANIMAL 6	MEAN
1	0.33	0.44	0.35	0.34	0.38	0.35	0.36
2	0.60	0.35	0.56	0.55	0.43	0.38	0.48
3	0.59	0.59	0.54	0.39	0.42	0.59	0.52
4	0.62	0.59	0.64	0.45	0.40	0.57	0.54

TABLE 2

TREATMENT	AV. MM^3 $O_2/10^6$ CELLS/HR.	PREDOMINANT TYPE OF CELL
One exudate	0.36	Mature neutrophiles, classes I, II, and III
Four exudates, 10 days apart	0.54	Young neutrophiles, class I, some metamyelocytes
Nucleic acid, followed by exudate	0.80	Metamyelocytes with coarse basophilic granules
Exudates in rapid succession	1.40	Myelocytic forms, some metamyelocytes

prominent. The animals were then injected intraperitoneally with 300 cc. of sterile isotonic NaCl, the exudate removed in the usual manner, and the respiration of the cells measured. These cells, which could not be other than juvenile forms, were found to have a greatly increased metabolic activity, the average O_2 consumption being 0.80 mm^3 $O_2/10^6$ cells/hr.

c. Lastly, these animals and some from the group which had produced four exudates at 10 day intervals were given further intraperitoneal injections of saline at 2 day intervals in order to push the marrow into a state of extreme stress. Under these circumstances, true neutrophiles soon disappear from the circulation and are replaced by relatively huge cells of myelocytic form, which have an O_2 consumption of from 1.2 to

1.6 mm^3 $O_2/10^6$ cells/hr., more than four times that of the average neutrophile.

These results are briefly summarised in table 2, which shows the average O_2 consumption of the cells of the peritoneal exudate after various forms of treatment, and also the predominant type of cell found in the circulation at the time of withdrawal of the exudate. The results clearly show that the O_2 consumption of the cells under consideration decreases with increasing maturity. Kempner (1936), and Ramsey and Warren (1932) have arrived at a similar conclusion in the case of erythroblast, reticulocyte, and erythrocyte.

The significance of this decrease in O_2 consumption of the increasingly mature cell, however, is far from clear, for although the myelocyte appears to have about 4 times the O_2 consumption of the mature neutrophil, cell for cell, the O_2 consumption may be more nearly equal when expressed per gram of wet or of dry tissue, or when expressed per unit of cell mass or volume. Again, the factor determining the magnitude of the O_2 consumption may be the cell surface (suggested by Ponder and Macleod, 1936), the size of the nucleus, or the amount of granulation (see Warren, 1934). Conceivably a number of factors are involved. This difficulty in interpretation occurs in all studies of cell metabolism, and in order to overcome it in the case of the white cell at least, we are engaged in a determination of physical and chemical attributes of leucocytes of various degrees of maturity, and in a correlation of these with the intensity of their metabolism.

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SIMILARITY OF EFFECTS OF ADRENALIN AND INHIBITORY SYMPATHIN ON INTESTINAL MOTILITY; SENSITIZATION BY DENERVATION[1]

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The similarity of adrenalin and sympathin has been shown by both chemical and biological tests (1, 7), but evidence is not available to determine if inhibitory sympathin is identical to adrenine or if it is adrenine modified by the effector before entering circulation. Sympathin of gastrointestinal origin produced by artificial stimulation of nerves, according to the theory of Cannon and Rosenblueth (3), must be either a mixture of two sympathins, ME and MI, or a mixture of ME, MI and M. Bacq's theory (1) postulates two substances, M and ME, rather than three. According to his theory inhibitory sympathin would be simply adrenine itself.

In a previous paper (9) we have noted the reflex production of intestine-inhibiting sympathin by abdominal nerves during the marked gastrointestinal inhibition elicited by rectal stimulation of unmedicated dogs. Additonal evidence has been obtained that inhibitory sympathin is produced by this method, and its effects on intestinal motility have been compared with the effects of constant adrenalin injections at various rates. It is believed that such comparisons will help to determine whether inhibitory sympathin is identical to adrenine. The use of low concentrations of adrenalin has resulted in demonstration of hypersensitivity of the denervated dog intestine to the inhibitory effects of adrenalin.

METHODS. Records of the activity of jejunal loops made into Thiry and Thiry-Vella fistulae were taken by the balloon-mercury-manometer method. Six dogs were used. Each of three dogs had two Thiry fistulae, one of which was denervated and the other innervated. One dog, in addition to having a denervated Thiry fistula, had one adrenal gland removed and the other demedullated. One dog had a Thiry fistula and its coeliac ganglia removed. One dog had a denervated Thiry-Vella fistula, one adrenal gland removed and the other demedullated, both vagi cut above the diaphragm, and the coeliac ganglia removed. Preparation of

[1] Made possible in part by a grant from the Wisconsin Alumni Research Foundation.

the fistulae and operations for interrupting nervous pathways were as previously described (9).

Rectal stimulation consisted of mechanical stimulation of the anal canal with a rubber catheter in order to elicit sphincter activity.

Perfectly regular injection rates were accomplished by means of an electric motor geared to a screw which moved the plunger of a 20 cc. syringe at the desired rate. The rate of injection could be varied between 1 and 4 cc. per minute, and adrenalin concentrations used were 1 to 500,000 and 1 to 250,000, allowing a variation in injection rate of 0.002 to 0.016 mgm. per minute. The two solutions were made by introducing 0.2 and 0.4 cc. of adrenalin 1 to 1000 (Parke, Davis & Co.) into 100 cc. volumetric flasks and filling the flasks to the mark with a 0.9 per cent NaCl solution having its pH regulated with phosphate to 4.9 to 5. Solutions of adrenalin, made by using this diluent, were allowed to stand in open beakers in the laboratory for 24 hours without showing discoloration. Solutions for the experiments were used within 2 to 3 hours after the time of preparation. Injection of the diluent alone at faster rates than any used in the experiments was without effect on the activity of the intestine.

A recent study (2) indicates that blood binds a part of adrenalin added to it. Therefore precautions were taken to prevent contamination of the adrenalin solution in the syringe with blood. This was made possible by the use of a three-way syringe stopcock attached to two syringes, one being used for the injection and the other to locate the vein. The needle inserted into the vein could be left in place throughout the experiment, the animal lying quietly on its side. The dogs showed no signs of knowing when the injections were begun or ended.

Results. *Additional evidence that inhibitory sympathin is produced during rectal stimulation.* In experiments with innervated and denervated Thiry fistulae in the same dog, rectal stimulation results in the inhibition of motility in both types. The latent period is 17 to 45 seconds longer in the case of the denervated intestine with fistula, which is due in part to circulation time. Since the innervated intestine with fistula is considered to be both a source of and an indicator for the neuro-hormone, the concentration in it should be greater than in the denervated. In one dog, however, the denervated intestine with fistula regularly showed greater inhibition than the innervated during rectal stimulation. In this case the greater effect in the denervated intestine with a fistula must have been due to hypersensitivity to inhibitory sympathin, which will be discussed later.

Since absolute proof of the humoral nature of the inhibition of a Thiry fistula rests on being certain of complete denervation, the nervous pathways to the loops were interrupted at more than one level. The gastrointestinal inhibitory effects of rectal stimulation are widespread (9), and

it is to be expected that abdominal sympathetic pathways which remain after coeliac ganglionectomy might permit the production of enough inhibitory sympathin to affect a fistula. Therefore, the coeliac ganglia were removed from each of two animals. One of these animals had both vagi cut above the diaphragm, one adrenal gland removed and the other demedullated, and a Thiry-Vella fistula that had already been denervated by the usual method of cutting the nerves in the mesentery and along the blood vessels. The other animal was vagotomized and had a Thiry fistula. The possibility of any nerves to the Thiry fistulae escaping these operations is exceedingly small, especially in view of the fact that either of the denervations alone left no physiologically detectable fibers. The animals withstood, without signs of pain, pressure much greater than that which obviously caused pain in innervated intestine. Each of these

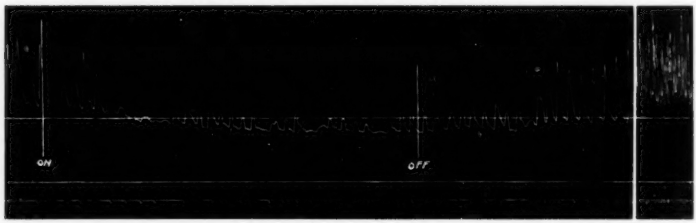

Fig. 1. Effect of rectal stimulation (between *on* and *off*) on the denervated Thiry-Vella fistula of a dog having both vagi cut above the diaphragm, one adrenal gland removed and the other demedullated, and the coeliac ganglia removed. Upper line marks the lowest tonus level before the stimulation or after recovery. Tonus during the stimulation reached a level of 11 mm. Hg below this. Detached part of record at right shows complete recovery 5 minutes following the end of rectal stimulation. Time in 5 second and 1 minute intervals.

animals, however, showed the usual inhibition of the intestine with fistula during rectal stimulation. Figure 1 shows a typical response of the doubly denervated Thiry-Vella fistula. Rhythmic contractions were almost completely eliminated, and the tonus fell 11 mm. of Hg below any level in the preceding or following normal. Both the onset of the inhibition and and the recovery from it were gradual. It appears from this that the lumbar outflow alone is capable of producing an effective amount of circulating inhibitory sympathin during rectal stimulation.

II. *Comparison of the effects of adrenalin and inhibitory sympathin on the denervated intestine.* The changes in motility of a denervated Thiry or Thiry-Vella fistula resulting from rectal stimulation have characteristic features. 1. There is a more or less gradual change from rhythmic contractions of normal amplitude to complete inhibition. 2. This change is associated with a decreased tonus. 3. If the rectal stimulation is con-

tinued sufficiently long (usually beyond 4 min.) the intestine gradually escapes from the inhibition. 4. After cessation of stimulation the amplitude of contractions steadily increases to a height which usually exceeds the normal. 5. Associated with the recovery of amplitude there is a rise in tonus which usually exceeds the normal. All of these effects could be duplicated in a given animal by means of a constant injection of adrenalin at the proper rate. Differences were most likely to occur at the onset of the inhibition which was earlier and sometimes less gradual after the intravenous injection of adrenalin. This was to be expected since the sympathin must have additional time to diffuse into the blood from the places of its production. The similarity of the response of the denervated intestine to adrenalin and to rectal stimulation (illustrated by figure 2) indicates that the substance produced at abdominal sympathetic endings during this procedure, so far as its effects on intestinal motility are concerned, could be adrenine itself. However, this fact alone does not afford

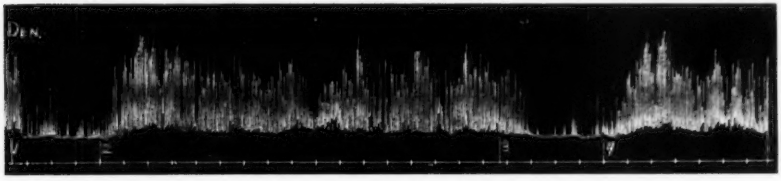

Fig. 2. Effects of adrenalin and of inhibitory sympathin on a denervated Thiry fistula. Adrenalin injection at a rate of 0.00042 mgm./kilo/min. was began at 1 and ended at 2. Rectal stimulation was begun at 3 and ended at 4. Time in minutes.

a basis for choosing between the direct and indirect action theories, for both the mediator and adrenalin may or may not have united with a substance in the effector.

Since the characteristic inhibitory effects of reflexly activated sympathetic nerves to the intestine can be entirely duplicated by adrenalin, the possibility that transmission of impulses from these nerves to the smooth muscle of the intestinal wall may be accomplished entirely by the production of adrenalin at the nerve endings has been demonstrated. This eliminates the necessity of assuming a dual transmission at these nerve endings.

Since the effects of rectal stimulation on the denervated intestine are so similar to those of adrenalin, it may be thought necessary to offer more conclusive proof that the inhibition is not due to adrenine from the adrenal medulla or from chromaffin tissue elsewhere. In varying the adrenalin injection from sub-threshold rates to rates several times as fast, it has been found that the degree and duration of the inhibition of the denervated in-

testine is proportional to the rate of adrenalin injection. This point is illustrated in figure 3. If it is assumed that adrenine is being produced reflexly from chromaffin tissue during rectal stimulation of normal animals, it is to be expected that at least the major part of the adrenine is coming from adrenal chromaffin tissue. Therefore, in view of the fact that the intestinal effects of adrenalin are proportional to its rate of entrance into the blood, the effects of rectal stimulation on the denervated intestine if due to adrenine from chromaffin tissue should be reduced by adrenal demedullation in proportion to the large per cent of chromaffin tissue removed by this operation. However, it has already been shown (9) that bilateral

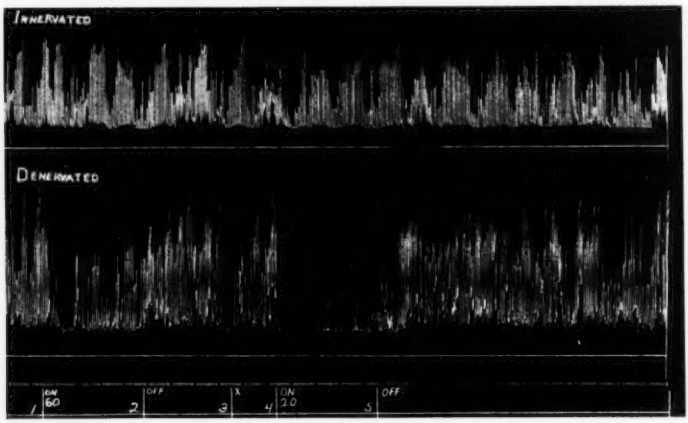

Fig. 3. Motility of an innervated and of a denervated jejunal Thiry fistula during adrenalin injections. Between 1 and 2 an adrenalin solution, 1 to 500,000, was injected at a constant rate of 1 cc. per minute. Between 4 and 5 the same solution was injected three times as fast. At 3 a few drops of the adrenalin solution were introduced in washing the needle. Dog weighed 14.5 kgm. Time in 5 second and 1 minute intervals.

adrenal demedullation does not reduce the degree of inhibition of the denervated intestine during rectal stimulation. Moreover, there is evidence that the recto-gastric and recto-intestinal reflex inhibition is a manifestation of the principle that when sphincters of the gastro-intestinal tract are activated the non-sphincteric musculature is inhibited. In view of current conceptions of the function of chromaffin tissue it is impossible to conceive of its being utilized in this mechanism.

The intestinal effects of inhibitory sympathin being similar to those of adrenalin, it is possible to determine the adrenalin injection rate necessary to produce a degree of inhibition comparable to that produced by

sympathin. This was not less than 0.00042 mgm./kilo/min. for dog 1 (fig. 2) and 0.00058 mgm./kilo/min. for dog 2.

III. *Relative sensitivity of the innervated and denervated intestine to adrenalin and sympathin.* Luco (4), in a study of the effect of adrenalin on the isolated, denervated rabbit intestine and on the non-pregnant feline uterus deprived of its sympathetic nerves, found that denervation renders these structures more sensitive to the inhibitory effects of adrenalin. He points out that the few papers relating to the subject of sensitization of inhibited structures by denervation are contradictory. If after denervation there is an increase in the sensitivity of smooth muscle to the inhibitory effects of adrenalin, one would expect it to become evident in these experiments where the innervated and the denervated Thiry fistulae are in the same animal and are made from adjacent segments of the intestine thus furnishing excellent controls. Moreover, the smooth muscle is receiving a normal blood supply and is not subjected to reflex inhibitory effects such as occur when the abdomen is opened. Finally, the animal is not under the influence of other drugs. Under these conditions the difference in sensitivity of the innervated and denervated intestine to the inhibitory effects of adrenalin was quite pronounced. An injection of adrenalin could be maintained at such a rate that the denervated Thiry fistula of a given animal showed complete inhibition of motility and decreased tonus while, at the same time, the motility of the innervated Thiry fistula was not affected. Using a regular injection rate, the minimum rate capable of producing a short period of complete inhibition in the denervated Thiry fistula could be doubled and, in one animal, trebled (fig. 3) and still not definitely affect the innervated intestine. Effects on the denervated intestine, however, were proportionately greater.

The changed sensitivity to the inhibitory effects of adrenalin on smooth muscle following denervation indicates that the response of the inhibited effector, as in the case of effectors stimulated by adrenalin, is not entirely dependent on the concentration of adrenalin in the blood. The response is in part dependent upon a physical or chemical factor in the effector.

The fact that in repeated experiments on one animal the denervated Thiry fistula was inhibited by rectal stimulation to a greater degree than the innervated Thiry fistula affords evidence that the denervation of the intestine also sensitizes it to inhibitory sympathin. This result may also be taken as additional evidence for the similarity of adrenalin and inhibitory sympathin.

IV. *Quantitative study of the sensitivity to adrenalin in denervated Thiry fistulae.* In dog 3 (14.5 kilo) 0.00014 mgm./kilo/min. of adrenalin injected at a constant rate was sufficient to produce a 40 second period of

complete inhibition of motility of the denervated Thiry fistula. Inhibition began to appear within 27 seconds after the beginning of the injection. The 1 to 500,000 dilution of adrenalin injected at a rate of 1 cc. per minute was further diluted by the blood to 1 to 750,000,000 if a minute volume of 1500 cc. is assumed for this dog (5). However, as explained in the next section, physiological effects on the intestine could be indirectly demonstrated with even lower concentrations.

An injection rate of 0.00020 mgm./kilo/min. was sufficient to produce a period of complete inhibition of the denervated Thiry fistula of dog 1. A rate of 0.00028 mgm./kilo/min. was necessary to produce this effect in dog 3.

Wada and Kanowoka (8) found that an adrenalin injection rate of 0.00020 to 0.00030 mgm./kilo/min. was the minimum effective dose for accelerating the rate of the denervated heart and for increasing the blood pressure of non-anesthetized and non-fastened dogs having their adrenal glands demedullated. It is not known if adrenal demedullation would change the adrenalin injection rate required to inhibit the denervated intestine. In any case the denervated intestine is among the most sensitive of adrenalin indicators in the dog. Its response is proportional to the amount of adrenine reaching it and it can be used innumerable times.

V. *Escape of the intestine from the motility-inhibiting effects of a continuous injection of adrenalin at a constant rate.* That the smooth muscle of the intestinal wall gradually becomes less sensitive to adrenalin as a result of its reception of adrenalin was indicated by two methods. First, an adrenalin injection rate several times as fast as the rate required to produce a short period of initial complete inhibition is not sufficient to maintain the complete inhibition for more than a few minutes. There occurs a gradual escape from the inhibitory effects. The same escape was seen in the denervated intestine during rectal stimulation when maintained beyond 4 to 5 minutes. Second, the adrenalin injection rate could be stepped up gradually from zero to double the threshold inhibitory dose without the inhibition occurring. For example, in dog IV the denervated intestine showed a period of complete inhibition when the injection rate was suddenly changed from zero to 0.0002 mgm./kilo/min., but, when the rate was increased by small increments from zero to 0.0004 mgm./kilo/min., using several minutes for the change, no period of complete inhibition occurred.

If we may assume, as seems logical, that during the continued injection of adrenalin at these constant rates the amount of adrenalin reaching the smooth muscle cell is being maintained and the adrenalin is not causing the production of oppositely acting hormones, then it appears that the rapid change in the degree of reaction on the part of the cell must be due to a change within the cell itself. Rosenblueth (6) has suggested that a

substance in the effector may be the factor determining the maximal response of the effector. These experiments indicate that a factor in the effector may possibly determine the length of duration of a submaximal response. At least it is clear that the development of refractoriness of an effector during the stimulation of its efferent nerves need not necessarily be explained by depletion of the immediate transmitting chemical but may be related to a subsequent step in the process of activation or inhibition of the effector.

SUMMARY

By the use of dogs each having both an innervated and a denervated intestinal loop with fistula, and by interrupting both the sympathetic and the parasympathetic pathways at two different levels additional evidence has been obtained that inhibitory sympathin is produced during rectal stimulation. An amount of sympathin sufficient to inhibit the denervated intestine could be produced after coeliac ganglionectomy. Sympathin production after these procedures would have to occur in connection with organs innervated from the inferior mesenteric ganglion.

All of the effects on intestinal motility of reflexly produced inhibitory sympathin were duplicated by injections of adrenalin at constant rates. Therefore, the possibility was indicated that the transmission of impulses from adrenergic sympathetic nerves to the wall of the intestine could be accounted for entirely by the production of adrenine at the nerve endings.

Direct evidence was obtained that a denervated Thiry fistula is much more sensitive to adrenalin than is an innervated Thiry fistula of the same dog. Estimated concentrations of adrenalin in blood between 1 to 500-000,000 to 1 to 1,000,000,000 have marked inhibitory effects on the motility of the denervated intestine.

The intestine, denervated or innervated, gradually becomes less sensitive to the inhibitory effects of adrenalin during the continued injection of adrenalin. Therefore, it is possible that escape from inhibition by adrenergic nerves is related to a step in neuro-effector transmission subsequent to the production of the mediator.

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ADRENALIN AND BLOOD LACTIC ACID: EFFECT OF EVISCERATION

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The experiments to be reported here will show that after evisceration (removal of abdominal alimentary canal with attached pancreas and spleen, and immobilization of the liver), intravenous injection of adrenalin no longer regularly increases blood lactic acid; rather, such increases as occasionally occur are much less than in intact animals and the usual and average effect is a temporary decrease in the blood lactic acid concentration.

METHODS. All of the work was done on cats, anesthetized with chloralose, 0.1 gram per kilo, subcutaneously.

The initial operative procedures as soon as anesthesia was complete consisted merely of insertion of necessary cannulae: 1, tracheal; 2, venous, in a superficial branch of the right femoral, for injection; 3, arterial, in both carotids, one for registration of blood pressure, the other for blood sampling. Following this a few of the animals were eviscerated at once; the majority, to provide normal controls, received an injection of adrenalin while still intact and were then eviscerated.

Intact animals. First injection: After a rest period of 30 to 45 minutes to permit stabilization following insertion of the various cannulae, the first "normal" arterial blood sample was taken; a second "normal" sampling, 15 to 20 minutes later, was followed immediately by commencement of adrenalin injection. This lasted always 5 minutes; immediately, and 5 to 10 and 30 minutes after its completion, additional samples were obtained. This permitted determination of the blood lactic acid level during the 15 to 20 minutes preceding injection, the immediate effect of the injection, and the effect persisting 5 to 10 and 30 minutes afterward.

Second injections: Using the last of these blood samples as a new "normal" this was followed at once with 16 animals of the above group by a second 5-minute injection, with samples again taken immediately, and 5 to 10, and 30 minutes following. These repeated injections are of interest here as controls for the reactions obtained after evisceration, usually with animals which had previously received a first injection as described above.

Eviscerated animals. Twelve cats were eviscerated as soon as anesthesia was complete so the injection they subsequently received was their first. These constitute a control against the possibility that the failure of adrenalin to raise blood lactic acid after evisceration in the remaining animals was due to the injection then received being their second; i.e., most of the animals of this group were eviscerated only after receiving an injection in their intact condition as just described.

Evisceration was through a ventral, median-longitudinal incision; ligatures were placed (in this order) on the coeliac, superior and inferior mesenteric arteries, the structures of the lesser omentum (hepatic artery, portal vein and bile duct), the cardia and the rectum. The alimentary tract with attached pancreas and spleen was removed; the liver though left in place was thus immobilized and excluded from participation in the remaining circulation. Placement of the arterial before the portal ligature diminished the amount of blood trapped in the eliminated viscera; usually, in addition, after arterial ligature and before tying the portal vein, warm Ringer solution, sometimes containing 1 per cent glucose, was injected peripherally into the mesenteric arteries until the viscera appeared free of blood. Presence or absence of glucose in this injection had no noticeable effect except to raise the blood sugar level from an average of about 130 for those that did not, to about 200 mgm. per cent, for those that did receive it. Whether or not due to this conservation of blood, blood pressure was uniformly good, averaging 130 and never below 90 mm. Hg, and the animals survived in apparently good condition for the duration of these experiments.

After an hour or more for stabilization following this operation, one (to avoid further excessive bleeding) or more often two "normal" blood samples were secured (in the latter cases, 15 to 20 minutes apart). Adrenalin was then injected as described above and the blood sampled again, immediately, and 5 to 10, and 30 minutes after injection was completed.

Adrenalin injection. Parke-Davis adrenalin chloride was used for injection, diluted immediately before using with neutral, 0.9 per cent NaCl solution to give concentrations of 0.001, 0.002, 0.003 and 0.004 mgm. per kilo per minute when injected 1 cc. per minute. Injection, into a superficial branch of the femoral vein, was always for 5 minutes (5 cc.) by hand from a 5 cc. syringe, carefully controlled as to this rate by injecting 0.1 cc. every 6 seconds.

Blood analysis. As already mentioned blood for analysis was secured from a large cannula, holding slightly over 2 cc., in one carotid artery. Exactly 2 cc. were removed by pipette to prepare a Folin-Wu tungstic acid filtrate; as much of this as necessary was used for lactic acid determination, in duplicate, by the method of Friedemann, Cotonio and Shaffer.

Results. *Intact animals.* The data on intact animals are presented

as the averages of 1, 35 initial injections, and 2, 16 second injections at rates of 0.001, 0.002 and 0.004 mgm. per kilo per minute for 5 minutes. The effects of each of these rates of injection though quantitatively slightly different were qualitatively alike and the total averages are alone of interest here as a control for the response after evisceration; since, then, over this same range, nearly identical effects were produced by each of the rates of injection used.

The average of the 35 initial injections into intact cats is shown as the lower of the two curves in the accompanying graph. It will be seen that

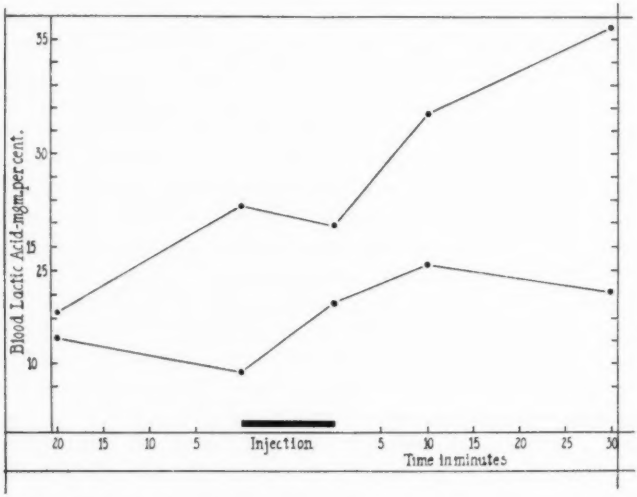

Fig. 1. The effect on blood lactic acid concentration of injecting adrenalin intravenously for five minutes at rates between 0.001 and 0.004 mgm. per kilo per minute. Lower curve: average of 35 injections in normal, intact cats. Upper curve: average of 33 injections into eviscerated cats. For other details see text.

at the time of injection under the conditions of these experiments (kind and amount and time after administration of anesthetic; previous operative trauma; etc.) blood lactic acid was slowly falling; 11.1 and 9.6 mgm. per cent being the average values at the beginning and end of the 20 minute normal period preceding injection. At the end of the 5-minute injection period, blood lactic acid is increased to 12.7, and 5 to 10 minutes later, to 14.3 mgm. per cent. During the subsequent 20 to 25 minutes the rate of decline is approximately what it was during the initial normal period, the value 30 minutes after injection being 13.1 mgm. per cent.

A second injection, beginning at the close of this half-hour recovery period, was made with 16 of the preceding 35 animals, 8 receiving 0.002

and the others 0.004 mgm. per kilo per minute. Starting from an average value of 11.2, blood lactic acid was increased to 13.8, 16.1 and 15.9 mgm. per cent, immediately, 5 to 10 and 30 minutes after injection, respectively. This is of interest in connection with the failure of adrenalin to increase blood lactic acid after evisceration, since 21 of the 33 eviscerated animals had already received an injection and objection might be made that this in itself would render a second injection ineffective. Such criticism is forestalled by this evidence that a second injection in intact animals produces practically the same increase in blood lactic acid as its predecessor.

So far only average responses have been described; it is of interest that adrenalin within the range of doses used failed to increase blood lactic acid only once in these 51 injections; of the remaining 50 responses the percentage increases and number of times occurring (in parenthesis) were: 5–10 (9); 11–20 (6); 21–30 (6); 31–40 (7); 41–50 (4); 51–60 (7); 61–70 (2); 71–80 (5); 91–100 (3); 187 (1). The number of experiments (again in parenthesis) with each of the rates of injection used (in mgm. per kilo per minute) were: 0.001 (8); 0.002 (22); 0.004 (21); and although the effect varied somewhat with dose, it is enough for the present purpose to have in mind merely the general range and average magnitude of response to rates of injection within these limits.

The effect of evisceration. Blood lactic acid level after evisceration. In 23 of the 33 experiments of this group, two "normal" blood samples were taken, 15 to 20 minutes apart during the second hour after evisceration and preceding injection; these established the slope shown for this portion of the curve for eviscerated animals in the accompanying graph; the average rate of this spontaneous increase is 0.235 mgm. per cent, per minute; and the average blood lactic acid level just preceding injection, 27.9 mgm. per cent.

The effect of adrenalin after evisceration. Referring to the graph it is evident that the immediate average effect of injecting adrenalin into these eviscerated cats is a decrease rather than (as in intact animals) an increase in blood lactic acid concentration; from the average value of 27.9 mgm. per cent, just preceding, blood lactic acid is reduced to 26.7 upon termination of the 5-minute injection; thereafter it again increases, but reference to the graph shows the values attained 5 to 10 and 30 minutes later are merely those that might have been expected from the uninterrupted continuation of the normal, spontaneous rate of increase which injection temporarily depressed.

This decrease of blood lactic acid during injection is not invariable; of the total 33 experiments, 3 showed no change; in 9 there were increases of 2, 3, 5, 12, 13, 16, 23, 31 and 55, average, +18 per cent; and in the remaining 21, decreases (with number of occurrences in parenthesis) of: 0 to −5 (5); −6 to −10 (3); −11 to −20 (7); −21 to −30 (2); −31

to −40 (3); −48 (1); average, −17 per cent. Thus 24 out of 33, or 73 per cent of injections, produced as an immediate effect no change or a decrease; and of the increases, only 3 out of 9 exceeded 20 per cent as compared with 35 out of 50 in the intact animals; and even if the predominating decreases are ignored, the average, +18 per cent, of the increases alone is approximately only one-half the increase of 32 per cent, attained at the end of injection in the intact animals.

This reduction of blood lactic acid concentration by adrenalin in these eviscerated animals is not proportional to rates of injection (within the range employed in this work) which were (with the number of experiments at each rate, in parenthesis) mgm. per kilo per minute: 0.001 (12); 0.002 (6); 0.003 (12); 0.004 (3); the average blood lactic acid level at the end of injection being, respectively, 96, 95, 93 and 96 per cent, of that at the beginning of it. Likewise, at each rate of injection the negative responses outnumbered the positive ones as follows (again, with the number of experiments in parenthesis):

0.001 mgm. per kilo per minute: no change (1); decrease (7); increase (2)
0.002 mgm. per kilo per minute: no change (2); decrease (3); increase (1)
0.003 mgm. per kilo per minute: no change (–); decrease (7); increase (5)
0.004 mgm. per kilo per minute: no change (–); decrease (2); increase (1)

In describing the effect observed in the control, intact animals attention was given to the fact that a repeated, second injection elevated blood lactic acid approximately as much as an initial one; consequently the preponderance of negative responses after evisceration is not to be attributed to the fact that the injection then made was in most cases the second these animals received, the first being made while they were still in an intact condition. A further check against this possibility is the evidence supplied by one group of the eviscerated animals, 12 in number, which received 0.001 mgm. adrenalin per kilo per minute; with these, this injection was the first they received and yet as has just been shown adrenalin was no more successful in raising blood lactic acid concentration with them than with the others.

Nor, finally, is the preponderatingly negative response after evisceration attributable to the remotely possible chance of increased excretion by the kidneys; in three experiments in which both kidneys were tied off at the time of evisceration, adrenalin reduced blood lactic acid concentration 3, 24 and 31 per cent.

Discussion. It is unnecessary to cite documentary support for the increase in blood lactic acid concentration produced by adrenalin in intact animals (for fairly complete bibliography see Ingvarsson, 1935). This reaction is now so well established that qualitatively it might have been

taken for granted. The data presented here are merely to provide quantitative controls related to the anesthetic, rates of injection and other experimental conditions peculiar to this particular work.

Measurement of: 1, arterio-venous blood lactic acid differences (Cori and Cori, 1929; Himwich, Koskoff and Nahum, 1929–30); 2, muscle glycogen concentration following injection (Cori and Cori, 1928; G. T. Cori, 1930; Corkill and Marks, 1930; Major and Mann, 1932; Masayama, 1932; Corkill, Marks and Soskin, 1934; Collip, Thomson and Toby, 1936; however see also Choi, 1928, and Firor and Eadie, 1930) or sympathetic stimulation (Corkill, Marks and Soskin, 1934); and 3, lactic acid formation in perfused (Janssen and Jost, 1925; Geiger and Schmidt, 1929; Roese, 1931) and isolated muscles treated with adrenalin (Wesselkina, 1931; Hegnauer and Cori, 1934) have furnished the basis for the prevailing opinion that the increased blood lactic acid concentration under the action of adrenalin is due to augmented glycolysis of skeletal muscle; and from this evidence there can be no doubt that under certain conditions adrenalin does increase muscle glycolysis and lactic acid formation. The only question of interest here is whether the increased blood lactic acid concentration observed in the normal animals of this work as a result of injecting adrenalin intravenously at rates between 0.001 and 0.004 mgm. per kilo per minute for 5 minutes is due to this cause.

Although muscle glycogen decreases progressively after hepatectomy (Mann and Magath, 1923; Bollman, Mann and Magath, 1925) it is apparently within normal limits up to five hours after evisceration in the spinal cat (Best, Hoet and Marks, 1926); and if this may be assumed for the eviscerated cats of this work it would appear from the minor and infrequent increases in blood lactic acid observed with them that the normal increase in the intact animal derives largely not from skeletal muscle but from the viscera.

It has been shown that lactic acid is a normal product of the metabolism of smooth muscle (Evans, 1925) and is increased under anaerobic conditions; and although following the work of Simpson and Macleod (1927) it has been increasingly assumed (Haarmann, 1932) that degradation of liver glycogen is always to glucose, there is considerable evidence of a sort that lactic acid may also be formed (Wissokowitsch, 1887; Macleod and Wedd, 1914; Elias and Sammartino, 1921; Gottschalk and Pohle, 1922; Sammartino, 1927; Orskov, 1932; Kato and Kimura, 1933). But much more relevant to the results being reported here, increases of blood lactic acid comparable in magnitude to those observed in the intact animals of this work have been produced (Griffith and Emery, 1935) by temporary (5 minute) clamping of the coeliac and mesenteric arteries or stimulation of the splanchnic nerve with adrenal removed; if the adrenal is present and adrenin is liberated (as evidenced by increased metabolic rate) blood

lactic acid is increased no more than by splanchnic stimulation or clamping the visceral arteries, alone.

It would also seem questionable whether the steadily increasing blood lactic acid following evisceration (or hepatectomy) which is seen in this work and has been often observed (Minkowski, 1886 and 1893; Asher and Jackson, 1901; Long and Grant, 1930; Anderson et al., 1931; Franke and Malczynski, 1935) is necessarily due to muscle glycolysis. Although it has been reported (Himwich, Koskoff and Nahum, 1929–30; Kimura, 1930) that venous blood from muscle contains more lactic acid than the arterial blood going to it even at rest, it has been suggested (Himwich et al.) that this when occurring may be due to the anesthetic; and others have not only failed to find such a difference at rest (Schneider and Widmann, 1929; Eggleton and Evans, 1930; Cook and Hurst, 1933) but have even failed to observe it at very considerable rates of work (Dresel and Himmelweit, 1929; Owles, 1930; Hefter and Okunew, 1931; Margaria, Edwards and Dill, 1933; Cook and Hurst, 1933). On the other hand there is abundant evidence that lactic acid from blood glycolysis varies reciprocally with carbon dioxide as a factor of normal acid-base equilibrium (Macleod, 1918 and 1921; Macleod and Hoover, 1917; Macleod and Knapp, 1919; Collazo and Morelli, 1926; Kilborn, Soskin and Thomas, 1928; Eggleton and Evans, 1930; Hayasaka and Itakura, 1931; Evans, et al., 1933; Cook and Hurst, 1933; Hartmann and v. Muralt, 1934; Hsu, 1935).

Evisceration in this work and as observed by others (Irving and Foster, 1930; Anderson et al., 1931) resulted in a progressive increase in pulmonary ventilation paralleling the increase in blood lactic acid. If the latter is made cause of the former there is not only the difficulty of accounting for its origin but there is the greater difficulty of explaining the actual decrease in concentration which has been seen usually to occur during adrenalin injection and which was accompanied by a parallel, marked decrease in pulmonary ventilation (the well-known adrenalin apnea). On the other hand if reflex or chemical stimuli, at present unknown, increase pulmonary ventilation after evisceration with consequent acapnia and compensatory increase of lactic acid from blood glycolysis, the actual decrease in blood lactic acid during injection, consequent upon the apnea and temporary retention of carbon dioxide, receives rational explanation.

SUMMARY

Intravenous injection of adrenalin into chloralose anesthetized cats at rates of 0.001, 0.002, 0.003 and 0.004 mgm. per kilo per minute for 5 minutes during the second and third hours after evisceration usually produces a temporary decrease in blood lactic acid concentration; and

such increases as occasionally occur are much smaller than are produced by corresponding rates of injection into intact animals.

It would therefore seem doubtful that increased blood lactic acid following injection of adrenalin *under the conditions of these experiments* derives in any considerable part from augmented glycolysis of skeletal muscle.

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A COMPARISON OF THE pH VALUES OF CORONARY VENOUS BLOOD AND OF BLOOD FROM OTHER VEINS

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In a previous communication (Moore and Greenberg, 1937) experiments were reported showing a marked fall in pH of coronary venous blood under conditions of myocardial ischemia produced by ligation of the coronary arteries. In these experiments it was difficult to obtain more than one sample of blood from the coronary vein. For this reason samples from the inferior vena cava were used as controls. Objection has been raised to the use of caval samples as controls, since apparently it has not been shown that under normal conditions the pH of coronary venous blood is the same as that of blood from the vena cava. To answer this objection we have compared in each of a series of animals the pH of blood from the coronary vein with that of blood taken from other venous sources.

The experiments were performed upon cats anesthetized with sodium amytal. The thorax was entered under artificial respiration. After wide incision of the pericardium the heart was tilted forward to expose its posterior surface. A blood sample of 1 cc. volume was then taken from the left coronary ("great cardiac") vein at a point distal to its junction with the coronary sinus. Since a valve guards the entrance of the coronary sinus into the right auricle, such a sample consists of undiluted coronary blood. The cava samples were taken from the lower thoracic inferior cava Ordinarily, within a time period of two minutes samples were taken from the coronary vein, from the vena cava, and from each auricle. The samples were collected under oil.

The pH determinations were made on plasma using the microquinhydrone electrode described by Cullen (1929). The exact procedure is outlined in a previous paper (Moore and Greenberg, 1937). Individual values given are the averages of duplicates or triplicates agreeing within 0.05 pH.

Average pH values were obtained by averaging the hydrogen-ion concentrations.

RESULTS AND DISCUSSION. In 12 animals the pH of the coronary venous blood varied from 7.31 to 7.54 and averaged 7.39. The value 7.54 was

unique and was apparently the result of over-ventilation by the respirator since a femoral vein sample taken before artificial respiration was instituted in this animal had a pH value of 7.35 whereas a second femoral sample taken later in the experiment had a value of 7.43. In 4 other animals in which such a preliminary femoral sample was taken for comparison with femoral blood drawn late in the experiment there was no evidence either of over-ventilation or of asphyxia.

The comparison of coronary with other venous blood gave the following results: 1, femoral venous blood averaged pH 7.31 in 5 animals in which coronary venous blood averaged 7.36, although in 2 of the animals the femoral blood was as alkaline as the coronary; 2, portal venous blood averaged pH 7.35 in 5 animals in which coronary venous blood averaged 7.38; 3, inferior vena cava blood averaged pH 7.38 in 8 animals in which coronary venous blood averaged 7.39; 4, right auricle blood averaged pH 7.40 in 6 animals in which coronary venous blood averaged 7.40; 5, left auricle blood averaged pH 7.46 in 5 animals in which coronary venous blood averaged 7.40 and in no case was the left auricle blood less alkaline than the coronary.

Under the conditions of the experiments the pH of the coronary venous blood was not significantly different from that of blood from the femoral vein, the portal vein, the inferior vena cava, or the right auricle. The oxygenated blood of the left auricle, however, was slightly more alkaline than the other samples.

As an additional study, in 5 experiments the lactic acid content was determined by the method of Mendel and Goldscheider (1925). Whereas the lactic acid level varied greatly from animal to animal there was no material difference between the content of the coronary and that of the caval blood, the coronary samples averaging 31.6 mgm. per cent and the vena cava samples 32.3 mgm. per cent.

It is concluded that under normal conditions the coronary venous blood and the inferior vena cava blood do not differ greatly in pH or in lactic acid content.

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POTASSIUM CHANGES IN THE FUNCTIONING HEART UNDER CONDITIONS OF ISCHEMIA AND OF CONGESTION

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A former series of experiments demonstrated a marked fall in the pH of the coronary venous blood when myocardial ischemia was produced by ligation of the coronary arteries (Moore and Greenberg, 1937). In view of the possibility that another effect of tissue asphyxia is a liberation of potassium, an additional group of experiments has been performed to determine the effect of ischemia upon the potassium content of the blood of the coronary veins. A supplementary series of potassium determinations during venous congestion is reported and serves as one form of control.

METHOD. The experiments were performed upon cats anesthetized with sodium amytal. The thorax was opened under artificial respiration.

In the ischemia experiments a ligature was passed beneath the stem of each coronary artery proximal to its first major branching. When both arteries are ligated at this level the ventricular musculature is deprived of any arterial blood. However, as described in previous communications (Moore and Greenberg, 1937; Moore, 1938), after both coronary arteries are ligated the continued contractions of the heart act mechanically to empty all blood from the coronary veins into the right auricle. Therefore, in order to obtain a sample of venous blood after a period of ischemia it was first necessary to confine blood in the coronary veins by obstructing the outflow into the right auricle. This was accomplished by ligating the small veins emptying directly into the auricle at its anterior border and then placing ligatures about the veins of the posterior aspect of the heart just proximal to their termination in the coronary sinus. Immediately these large veins were tied the previously placed arterial ligatures were tightened. With this method it was possible, after the ischemic heart had functioned for the desired period, to obtain one satisfactory sample of blood from the left coronary vein (0.2–0.5 cc.) for comparison with vena cava blood drawn immediately after the coronary sample. In practice, the coronary sample was taken when the occurrence of ventricular fibrillation

or of extreme ventricular dilatation indicated that the heart had ceased to maintain the circulation, wherefore the animal was moribund.

The effects of venous congestion were studied in a separate series of animals in which only the veins were ligated. In these experiments the coronary veins became greatly engorged and blood samples of large volume could be obtained.

The potassium content of the samples was determined by the method of Truszkowski and Zwemer (1936, 1937). The accuracy of the procedure was checked by analysis of solutions of potassium salts of known concentration and by determination of the percentage recovery of potassium added to various blood samples. Satisfactory results were obtained with quantities of blood as small as 0.2 cc. Duplicate determinations consistently agreed within 5 per cent.

Results and discussion. In 4 preliminary experiments, immediately the thorax was opened a sample of coronary venous blood was obtained by the method outlined by Moore and Dennis (1938). The potassium content of these coronary venous samples varied from 18.8 to 24.5 mgm. per cent in the 4 animals and averaged 21.7 mgm. per cent, whereas the potassium content of blood from the inferior vena cava of the same animals varied from 19.6 to 24.5 mgm. per cent and averaged 22.2 mgm. per cent. The greatest difference in the potassium content of coronary and caval samples in any animal was 1.8 mgm. per cent, a difference which is not considered significant. It was concluded that under the conditions of the experiments it is permissible to use caval samples for obtaining normal or control values.

In each of 9 animals a sample of coronary venous blood was taken after a period of myocardial ischemia varying from 5 to 9 minutes and averaging 7 minutes. The potassium content of these samples varied from 29.4 to 43.6 mgm. per cent and averaged 38.1 mgm. per cent. Vena cava samples taken from the same animals immediately after the coronary samples had a potassium content varying from 19.2 to 29.7 mgm. per cent and averaging 24.8 mgm. per cent. In every animal the potassium content of the coronary venous blood had risen considerably above that of the blood from the inferior cava, the least increase being 5.4 mgm. per cent in animal 32 after 8 minutes of ischemia and the greatest increase being 22.4 mgm. per cent in animal 31 after 6 minutes of ischemia. The average increase in the 9 animals was well over 50 per cent.

In each of 5 other animals coronary and caval samples were taken after a twenty-minute period of congestion produced by ligating all the major cardiac veins. The potassium content of the coronary venous samples in these animals varied from 17.2 to 22.5 mgm. per cent and averaged 19.8 mgm. per cent whereas the potassium content of the caval samples in the

same animals varied from 18.9 to 21.1 mgm. per cent and averaged 19.5 mgm. per cent. That the greatest difference in potassium content of coronary and vena cava blood was 1.8 mgm. per cent demonstrates that the myocardial congestion did not effect any liberation of potassium sufficient to be detected in the coronary venous blood.

Since operative shock leads to a liberation of potassium (Zwemer, 1937), it seemed desirable to determine whether any increase in the potassium content of the vena cava blood occurred during the progress of these experiments. Therefore, in 5 animals of the ischemia series a preliminary vena cava sample was taken immediately upon opening the thorax. These samples showed an average potassium content of 22.0 mgm. per cent whereas the caval samples taken at the termination of the experiment in the same animals showed an average content of 24.5 mgm. per cent. These results suggest that during the interval between the two samples—a period averaging 40 minutes—the operative procedure had led to a slight increase in the blood potassium. However, in 4 of the congestion experiments a similar comparison was made and in this instance the samples taken immediately the chest was opened had an average potassium content of 20.0 mgm. per cent whereas the terminal samples taken after an interval averaging 30 minutes had an average content of only 19.5 mgm. per cent. In these latter experiments, although the heart became so congested that it assumed a purplish hue and literally "wept" from transudation before the termination of the experiment, the beat remained slow and regular and an active corneal reflex indicated that the animal continued in good condition. In contrast, in the animals in which the coronary arteries were tied the corneal reflex was lost after a few minutes and at the time the experiment was concluded erection of hair and dilatation of the pupils suggested that the circulation had failed to the point of asphyxia. Since it was only in these ischemia experiments that the potassium content of the systemic blood underwent an elevation, it is reasonable to assume that the increase resulted from asphyxiation of the animal secondary to the failing heart and did not arise from the trauma of the operative procedure.

In recent years considerable interest has been attached to the study of ionic changes in muscle under various conditions. Although many investigators have studied changes in potassium, we know of no previous investigation of the potassium concentration in the coronary venous blood either under normal conditions or with an ischemic myocardium. However, several previous reports are of particular interest in this regard. In a series of papers Fenn and Cobb (1934, 1935, 1936 and 1937) have pointed out that the potassium content of muscle is lower following exercise and that during the recovery period the potassium re-enters the muscle. Kehar and Hooker (1935) have shown that during ventricular fibrillation

the perfused heart loses potassium. Baetjer (1935) has found that a restriction of the arterial blood supply leads to a loss of potassium from resting skeletal muscle.

The experiments reported at this time demonstrate that there is an increase in the potassium content of the coronary venous blood under conditions of myocardial ischemia. The rise in potassium in the coronary venous blood must indicate a loss of this element from the heart muscle. This escape of potassium from the laboring muscle is a result of the deprivation of arterial blood. It is quite possible that the liberation of potassium from the asphyxiated muscle cells is related to the marked accumulation of acid metabolites in ischemic muscle demonstrated by Moore and Greenberg (1937).

The escape of potassium after ligation of the coronary arteries is of interest in connection with Ringer's demonstration (1882) that depletion of the potassium in heart muscle leads to a cessation of contraction. It is possible that in clinical cases of myocardial ischemia the loss of potassium from the muscle fibers constitutes one factor contributing to the eventual failure of the heart. This suggestion has also been made by Harrison, Pilcher and Ewing (1930) on the basis of their studies of congestive heart failure in man.

In regard to the magnitude of the increase in potassium in the coronary venous blood it should be remembered that in the experiments reported, in order to obtain the blood sample from the veins of the ischemic heart, it was necessary to prevent their collapse by ligating them proximally immediately before ligating the arteries. The venous sample consisted, therefore, of normal venous blood trapped in the veins at the moment of ligation, admixed with blood from the ischemic heart muscle, added to the sample as the heart contracted after ligation of the arteries. In such mixtures the first ingredient serves to dilute the second and an increase of 50 per cent in the potassium content indicates that a still greater change would have occurred had the sample been undiluted. In the tissue spaces of the laboring muscle the increase may have been very great indeed.

One of us has reported experiments in intra-arterial injection which demonstrated that pain-endings are sensitive to the potassium ion when it is present in the concentration represented by 150–160 mgm. per cent (Moore, 1934). Whether in the ischemic heart such a concentration is attained in the intercellular spaces about the nerve-endings is not known. Moore and Greenberg (1937) have shown that the acid change in ischemic muscle is adequate to produce pain. The possibility of a similar stimulus arising from a liberation of potassium should be kept in mind.

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ON THE METABOLISM OF GLUCOSE

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A simple hexose sugar such as glucose is more reactive in an alkaline than in an acid medium. While it is true that glucose undergoes an intramolecular rearrangement in acid, its carbon chain is broken only after prolonged heating with strong acids. In an alkaline solution on the other hand it gives rise to other glucose-like sugars, absorbs measurable amounts of oxygen, and even at ordinary temperatures is broken up into smaller fragments. Unlike glucose, glycogen is quite stable in alkali but is unstable and converted into glucose in acid solutions. Although these differences in the reactions of glucose and glycogen are well known and the regulation of the concentration of the blood sugar and the ability to assimilate glucose were ascribed to similar chemical changes by F. P. Underhill and by L. J. Henderson over twenty years ago, their signficance in explaining and correlating many of the facts of carbohydrate metabolism has not been sufficiently emphasized by subsequent workers (1).

If the instability of glucose in alkali is duplicated in the tissues of an organism, then the ability of an animal to utilize glucose should vary with any variation in its total alkalinity. And if the instability of glycogen in an acid solution is manifest also by glycogen within the tissues, then the power to synthesize and to store glycogen should decrease in an animal intoxicated with acid. If this holds true then we should find *that a state of hyperglycemia results whenever there is a general increase in the total acidity of a whole organism or a local increase in the total acidity of a single tissue, such as the liver.*

The first proof of this theorem was found in the work of Hofmeister, who in 1899 discovered that an alimentary glycosuria was induced more easily in a fasting animal than in one on a normal diet. And the first to recognize the rôle of acid in producing a hyperglycemia was MacLeod, who attributed the high blood sugar of asphyxiated dogs to the acids carried in their blood. The first direct proof of this postulate was found in the

[1] The conclusions presented here developed from studies which were made possible by a grant from the Committee on Scientific Research of the American Medical Association.

work of Elias (2), who discovered that the intravenous injection of acids into dogs resulted in hyperglycemia and glycosuria. Elias believed that the acid caused a discharge of glycogen from the liver and suggested that an acidosis exerted a similar action in diabetes. Furthermore he said that the starvation diabetes of Hofmeister was due to the reduced alkalinity of the blood which accompanies fasting.

These findings have been reproduced in other ways. Thus a hyperglycemia resulted when an acid intoxication was induced by generalized convulsions, by maintaining dogs in an atmosphere rich in carbon dioxide, and by feeding acidifying diets (3). The acidosis which accompanies many infectious diseases, skin diseases, uremia and nephritis (4) has also been found to be associated with hyperglycemia. Following the administration of both pituitrin and thyroxine and in patients suffering from hyperthyroidism, there is hyperglycemia with an attendant increase in acidity, as evidenced by an increase in the hydrogen ion concentration and the lactic acid of the blood (5). Likewise a loss of liver glycogen and an increase in blood sugar are coupled with a low alkali reserve (6) in cases of surgical shock.

T. Araki and H. Zillessen (7) have shown that any condition which results in an inadequate supply of oxygen to the tissues of an organism, whether due to alterations in the composition of the blood or the respired air, or to pathological changes in the respiratory or circulatory systems, makes for an abnormal accumulation of acid in the organism and the excretion of lactic acid in its urine. And in the accumulation of acid which follows an insufficient supply of oxygen we find an explanation for the hyperglycemia which is associated with any oxygen deficiency, whether it be due to carbon monoxide poisoning, pneumonia or anemia, to arteriosclerosis, circulatory failure or the decreased flow of blood after injections of pituitrin, or to the use of such drugs as the narcotics or anesthetics. Proof that these effects are not directly due to the lack of oxygen, but to the acidosis which is a consequence of the oxygen deficiency, is to be found in the work of Cordier cited above. He found that dogs develop a hyperglycemia when they breathe air which contains high concentrations of carbon dioxide even when it is mixed with normal amounts of oxygen.

Just as hyperglycemia attends any prolonged generalized acid intoxication, so a local accumulation of acid in a single tissue results in increased glycogenolysis in that tissue. When this occurs in such a great storehouse of glycogen as the liver, a demonstrable hyperglycemia follows. Thus, if the blood supply to the liver is shut off for a time by a ligation of its arteries, the venous blood, after the ligatures are removed, is found to have a diminished capacity to neutralize acids and to contain increasing amounts of lactic acid and glucose (8). The injection of adrenalin is followed by a constriction of the hepatic venules and a stasis of blood in the liver.

Hence the hyperglycemia which ensues may be attributed to changes which are similar to those which result from ligation of the hepatic arteries. A like mechanism explains the hyperglycemia which follows emotional upheavals, the sugar puncture of Bernard and the use of pituitrin (9).

Finally we see how the physiological increase in glycogenolysis which occurs in fasting animals is accompanied by circulatory changes which make for an increase in the acid content of the liver. For during digestive rest there is a decrease in the volume of arterial blood and an increase in the amount of highly venous portal blood which enters the organ (8). The increase in the total acidity of the liver which thus results from its inadequate oxygenation is manifest also by a general increase in the total acidity of the whole animal, and the most active conversion of glycogen into glucose occurs during this "acid tide."

The acceleration in the rate of glycogenolysis which occurs with an increase in acidity is greater in the liver than in the other tissues. The glycogen in the skeletal muscles, for instance, is not hydrolyzed as readily as that in the liver, perhaps because the water in muscle is not as labile and because such tissue is not as vascular and therefore is not exposed to external chemical stimuli as effectively as liver tissue. But the same forces govern glycogenolysis in the muscles as well as in the other tissues, and we find that an increase in acidity is always accompanied by greater hydrolysis of the glycogen in all of the tissues and an elevation of the concentration of glucose in the blood. We shall see now that the converse is true and that any *general or local increase in the alkalinity of an organism is attended by hypoglycemia and an increase in the ability of the animal to utilize sugar.*

The stability of glycogen dissolved in an alkaline solution is evidenced also by glycogen which is stored in the liver, for while the postmortem conversion of liver glycogen into glucose is accelerated by small amounts of acid, it is retarded or stopped by alkali (8). Proof that this is a general reaction, which occurs also in the living organism, is found in the studies of Bonnanno and Costa and of Kamamura (10), who reported a decrease in the blood sugar and an increase in liver glycogen with any increase in the alkalinity of an organism; of Elias, who found that the administration of alkali prevented the hunger diabetes of Hofmeister and the hyperglycemia which follows injections of adrenalin; and of Underhill and of Murlin who reported an increase in sugar tolerance after the ingestion of sodium bicarbonate (11). The increase in alkalinity which accompanies forced breathing, epilepsy, prolonged vomiting and pyloric obstruction (12) was likewise found to be associated with decreased glycogenolysis and hypoglycemia.

It has been said that "the liver can only form glycogen if it be supplied with sufficient oxygen (8)." This may be restated: The liver can form

glycogen only when it is sufficiently alkaline, if we recall the discovery of Zillessen and Araki that adequate oxygenation makes for decreased acidity. Mathews calls attention to the fact that during digestion when the formation and storage of glycogen is at its height, vasodilatation of the splanchnic vessels occurs and the portal blood becomes almost as rich in oxygen as the arterial blood. It is significant that during the period in which the most active storage of glycogen takes place we observe not only a general reduction in the total acidity of the animal, the alkaline tide, but also the circulatory adjustment which produces a greater alkalinity of the liver. Perhaps future investigations will show us that there is a similar but prolonged dilatation of the splanchnic vessels with a consequent increase in the activity of the liver in those cases of glandular insufficiency which evidence an increased sugar tolerance and hypoglycemia.

While there is a loss of glycogen from the liver and skeletal muscles of an animal intoxicated with acid, whether from diabetes, carbon dioxide poisoning, starvation or after the use of adrenalin, an accumulation of glycogen occurs in the heart muscle in these conditions. The explanation for this increase in the glycogen store of the heart, which at first hand seems at variance with our postulate that a gain in alkalinity is necessary for the formation and deposition of glycogen, is to be found in the changes which occur in the coronary circulation following the injection of adrenalin or any intoxication with acid. For after the use of adrenalin or any increase in the acidity of the blood there is a vasodilatation of the coronary vessels and a sequential increase in the circulating blood and alkalinity of the heart muscle. Likewise we attribute the increase in glycogen around areas of necrosis to an accumulation of the ammonia which forms whenever tissues suffer autolysis.

The rôle of ammonia in stimulating anaerobic oxidation in cells (13) and in lessening acidity and hence checking glycogenolysis in the liver has been recognized (14). We attribute the changes in sugar metabolism which occur after the removal of the liver to the accumulation of ammonia which follows hepatectomy. The progressive loss of blood sugar which ensues after the extirpation of the liver has been credited to the loss of the great store of glycogen in the organ. Evidence that the hypoglycemia is due to some other cause is to be found in the fact that while the symptoms of hypoglycemia may be relieved and life prolonged for a time by the intravenous injection of glucose, the animal dies approximately twenty-four hours after the hepatectomy and often with enough glycogen in its muscles to maintain the blood sugar at its normal level for a long time. Moreover the removal of the liver from a depancreatized dog results in an increase in the respiratory quotient (15). We hold that the progressive and fatal hypoglycemia is due to the reduction in the total acidity which

attends the accumulation of ammonia in the blood and tissues after removal of the liver, and that death is due to the inability of the animal to maintain an adequate blood sugar in the face of the consequent reduction in the rate of glycogenolysis and of the increased sensitivity of its glucose to oxidation. It is of interest to recall that the symptoms ascribed to alkalosis, of tetany and convulsions, are also those observed in hypoglycemic states.

It has been shown that alkalosis promotes and acidosis hinders the utilization of carbohydrate and that the decomposition of sugar by bacteria occurs more readily in an alkaline than in an acid medium (16). Furthermore it has been shown that the tissues of depancreatized animals are able to consume glucose when they are perfused with Locke's solution (17). Locke's solution contains sodium bicarbonate, so we have in this instance a demonstration of the ability of a tissue to utilize sugar when it is supplied in an alkaline medium. It is well known that the loss of glycogen in a fatigued muscle is accompanied by the formation of lactic acid. And the reappearance of glycogen and the disappearance of lactic acid in the resting muscle has been cited as evidence that muscle glycogen is resynthesized from lactic acid. It seems likelier, in view of the works which have been cited, that glycogen is reformed from blood glucose after lactic acid and other acid metabolites are neutralized and when the muscle is restored to its normal alkaline reaction.

We shall see moreover that an increase in the total alkalinity of an organism is characterized by an increase in its ability to utilize oxygen as well as glucose. The influence of acid and alkali on cellular metabolism has been recognized by Mathews, who said that "the respiratory oxidation of a cell is wonderfully affected by a rise or fall of acidity. By an increase in acidity it is checked; by a decrease in acidity it is greatly stimulated." A recent proof of this was found in the works of Kodera and Achi who reported a decrease in the intramuscular consumption of oxygen and carbohydrate in dogs following the intravenous injection of hydrochloric acid, and of Campbell and Wuth (18) who found that an increase in the consumption of oxygen and in the metabolic rate occurred after the ingestion of alkali. The increase in total alkalinity which results from excessive breathing was found to be accompanied by a fall in the blood sugar by Jacoby (19), an increase in the intramuscular consumption of sugar and oxygen by Kodera (20), and by an increase in oxygen consumption at the expense of readily oxidized foodstuffs, by Campbell.

Sufficient evidence has been presented to show that the in vitro reactions of glucose and glycogen with acid and alkali are duplicated in vivo, and that we may state the following conclusion as a description of a constant and general mode of behavior: *That glycogenolysis varies directly and glucolysis varies inversely with the total acidity of a single tissue or a whole*

organism. And while no new data have been presented, we offer this study as an attempt to correlate many facts of the physiology of glucose and glycogen under this general conception.

The increase in the ability of an organism or single tissue to utilize glucose with an increase in its total alkalinity and the profound changes, the loss of glycogen from the tissues and the decreased ability to store and to utilize glycose whenever there is an accumulation of acid, teach us anew the importance of the recognition and treatment of an acid intoxication (21). For a slight acidosis at the onset of an infection, following an anesthetic, nephritis or a circulatory difficulty, may become intractable and disastrous when the consequently lowered sugar tolerance (and the coarsening of tissue fat) results in a further acid intoxication through the incomplete combustion of fat. These effects of acidosis on the metabolism of glucose show us why an author has written "I do not recall a single patient in whom it was difficult or impossible to hold the urinary acidity below the turning point of methyl red who did not die (22)."

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THE EFFECT OF BRIEF CURRENTS ON AXONS, ESPECIALLY IN RELATION TO THE POSTULATED NONCONDUCTED RESPONSE

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In a recent paper, Katz (1937) describes a change in the temporal configuration of latent addition curves derived from frog's nerve which becomes demonstrable when a conditioning shock is increased to a strength exceeding half threshold, and concludes that this alteration is referable to the appearance and growth of a nonconducted nerve response which adds its effect to that of the stimulus. According to this view the electrical record obtained from the cathode, as the strength of the shock is increased, consists at first merely of an electrotonic potential to which is added, at about 50 per cent of threshold, a nerve response which at first is minimal in length and strength but increases until at threshold it becomes long enough and strong enough to propagate itself.

A similar hypothesis had been considered by Blair and Erlanger (1936a) in connection with studies on the process of excitation by brief shocks and had been discarded, but since the question has been reopened it is here subjected to a reinvestigation. Additional results have been obtained which confirm the prevailing view relative to the nature of the local excitation process in nerve and further elucidate the mechanism of stimulation by brief shocks.

METHODS. All experiments were done at room temperature on the most irritable fiber in excised phalangeal nerves of the green frog (*Rana pipiens*). The amplified spike of the fiber served as an all-or-nothing index of response. The amplifying and electron oscillograph apparatus employed has been described in previous publications (Erlanger and Gasser, 1937). A nerve chamber[1] (fig. 1) has been constructed which permits of the insertion of ball and socket holders into the walls and top at any desired point. Glass tubes passing through the centers of the balls can be adjusted from outside the chamber, and serve as electrode and nerve holders. The electrode connections run through these tubes and are protected by them to the extent that the electrode resistance to

[1] Designed and constructed by John H. Zimmer.

ground is usually in excess of 50 megohms. Small calomel half-cells of a new design (fig. 1,*E*) have proved very satisfactory as electrodes.

Condenser discharges through a grid-controlled gaseous discharge tube (RCA 885) have been employed for stimulation. The tube fails to conduct when the condenser discharges to about 10 volts, or, under usual conditions, when the condenser reaches 90 per cent discharge. This behavior eliminates the last 10 per cent of the condenser discharge which with usual methods of stimulation approaches zero asymptotically and therefore adds directly to the latent addition effect of the shock. By interposing

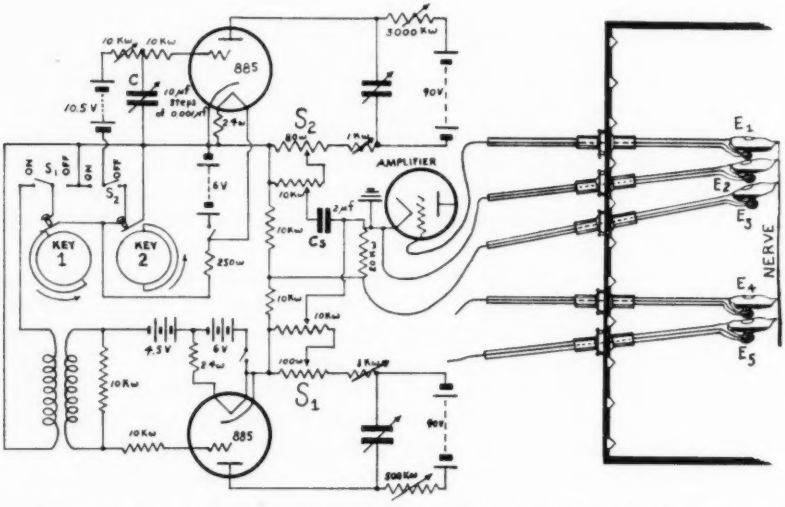

Fig. 1. Wiring diagram of a stimulating device (designed by H. B. Peugnet) and of electrode arrangements for stimulation at the lead electrode with paired truncated condenser discharges of variable strength and duration (the polarity of the biasing battery for the tube delivering S_2 should be reversed). The interval between shocks varies directly as the capacity of the condenser *C*. Electrode E_2 is common to the amplifying and stimulating circuits.

the large series condenser, *CS*, the shocks can be made diphasic, with a very brief, intense stimulating phase and a long opposite phase of very low intensity. To reduce polarization the testing shock usually has been made diphasic. The duration of the shocks may be changed independently by adjustment of the condenser size or of the discharge resistance. By adjustment of secondary potential dividers, the calibrated potential dividers in the discharge circuits are set so that full scale delivers a threshold shock. Under these conditions shock strengths may easily be read to 0.5 per cent of threshold.

Intervals between shocks are determined by the capacity of a variable condenser in a standard delay circuit. The range of the delay circuit is from 10 microseconds (μsec.) to 100 milliseconds (msec.) in steps of 10 μsec. Oscillograms with a sweep of 1 mm. per μsec. show no variation with a fixed delay time.

The apparatus operates as follows: A cam making 40 revolutions per minute synchronizes sweep and stimulation. The opening of key *1* delivers the first, the conditioning, shock (S_1) through the activation of an 885 tube by a break induction shock. At the same time the voltage on the delay condenser, C, begins to rise. When a critical voltage is attained (the time depending on the capacity) the grid loses control and the second, the testing, shock (S_2) is delivered. To prevent the tube from discharging a second time when the stimulating condenser again becomes charged, key *2* reëstablishes grid control.

The duration of S_1 has been determined by an RC product of 4.3 μsec., that of S_2 by an RC product of 25 μsec. In some of the earlier experiments the induction shock here used to activate the first tube was applied directly to the nerve and served as S_1.

RESULTS. Two general groups of experiments have been performed. The first was planned with a view to ascertaining whether there is a local nonconducted response; the second with a view to ascertaining whether the strength of the conditioning shock qualifies latent addition determinations, and, if so, whether the views on nerve excitation formulated previously by Blair and Erlanger (1936a, 1936b) require revision.

I. (a) *Is there a nonconducted response?* A local response of the kind described by Katz should be all-or-nothing for any length of nerve responding and, as admitted by Rushton (1937), should therefore be followed by a refractory period. A nerve impulse conducted from a remote point to a region which has just responded locally, should find the nerve refractory there and be blocked.

Therefore in one series of experiments a just subthreshold shock, S_1, was imposed on the nerve with the cathode at E_2, the anode at E_3 (fig. 1). E_2 was connected directly to the ground without the use of any balancing device. Since E_2 is common to both the lead and the stimulating circuits any change in the character of the response at E_2 would be recorded. S_2, delivered through E_4 and E_5 produced a conducted diphasic response. Under these circumstances no temporal separation of the two shocks could be found which would cause S_1 to block or modify in any way the conduction time or configuration of the spike conducted from E_4. S_1 was then increased in strength to the point where, with the spontaneous changes in the excitability of the fiber, responses to it were infrequent. Under these conditions the spike initiated at the lead had essentially the same height and configuration as those conducted from the remote point. Whenever

a response was initiated at the lead, the fiber was refractory and the response from the remote point was blocked. When S_1 failed to stimulate at the lead, the record of the conducted response was unmodified by the just subthreshold shock, S_1.

Blair and Erlanger (1936c) have described an experiment which indicates that under exceptional conditions a nerve impulse may be propagated across a short nonconducting locus. It is therefore conceivable that the action potential from a remote point might be sufficiently intense, and the length of the local response (and refractoriness) so short, that the impulse could "jump" the refractory region. However, the subthreshold shock is delivered through the lead electrode, and therefore the interposition of a refractory stretch, no matter how short, would alter the amplitude and shape of the conducted spike. Since the conducted spike comes through unaltered it may be concluded that a subthreshold shock does not produce a refractory state.

To render the conditions even more exacting the nerve in some trials was depressed at the lead electrode by means of anodal polarization so that about half of the impulses from the remote point were blocked there. Now the nerve impulse just sufficed for conduction, yet a just subthreshold shock applied at the lead electrode through the polarizing circuit did not at any temporal separation of shock and impinging spike alter the relation of unconducted to conducted responses. It is inconceivable that the nerve impulse, depressed to this extent, could be propagated across an unresponsive locus. A subthreshold shock does not produce refractoriness to conduction; the local response it produces is not like that elicited by a threshold shock.

(b) *Is there a local electrical change not directly referable to the applied potential?* Katz, with a slow, sensitive galvanometer and very rapid stimulation, has observed an electrical change in nerve which begins to appear when the shock reaches half threshold, and states that Hodgkin has obtained convincing records of local nonconducted action potentials from isolated crustacean nerve fibers. We have therefore compared at the stimulated locus the electrical record elicited by threshold shocks with that obtained with half threshold stimulation.

When the lead electrodes, E_1 and E_2, figure 1, are adjusted to a separation which is just adequate to record the maximum monophasic spike, and the separation of the stimulating electrodes, E_2 and E_3, reduced so that the decreased resistance permits stimulation with a relatively low voltage, the common electrode E_2 may be frankly grounded. In spite of the fact that E_2 is common to both circuits, and that no shock balancing circuit is employed, the amplitude of a threshold shock escape may be no higher than the spike from a single fiber. With frankly diphasic leads, probably because the spike is lower, and because core conduction permits electrotonic spread of the shock to the grid electrode, such a favorable spike amplitude in relation to shock escape has rarely been obtained.

The nerve was stimulated at the lead by means of a truncated condenser discharge with an RC product of 4.3 μsec., made diphasic as described above by the introduction of a large series capacity. Three records obtained with a threshold shock are shown in figure 2A. Record *1* was obtained when, due to a spontaneous variation in the excitability of the fiber, the shock failed to stimulate. This record serves as a base line for measurement of the spikes, *2* and *3*. The time from the start of the 4.3 μsec. shock, to the earlier spike (*2*) was 156 μsec., to the latest (*3*) 268 μsec. Then, in order to minimize the local response, the shock strength was reduced to half threshold, and the amplification doubled so as to make the shock component comparable under the two conditions, and record B*2* was obtained. In figure B, it is superimposed on the record of the threshold shock of A, for comparison. In spite of the high noise level with

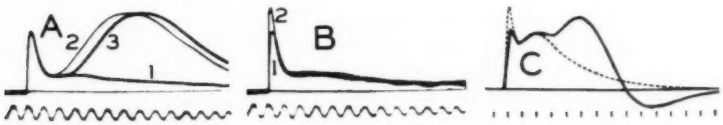

Fig. 2. A. Three superimposed responses to just threshold shocks, all of the same strength. The shock had an RC product of 4.3 μsec. and was diphasic, so that the current was reversed, but of low potential, after about 20 μsec. 1, "shock escape" alone; 2 and 3, spikes from the same axon. The records demonstrate the constancy of the "shock escape" and the variability of the shock-response time in different trials.

B. Escape, 1, of the threshold shock shown in A1 superimposed on an escape, 2, secured by reducing the shock strength to half and doubling the amplification. The latter is the higher.

C. Solid line: A late diphasic spike. Dotted line: "Escape" from the shock reduced to half threshold with amplification doubled. Traced from records.

Time: 10,000 cycles. All records are from the locus of stimulation.

an amplification twice that which was needed to record spikes of single fibers, the comparison shows that the two records differ only in their earlier parts. The stronger shock in B*1* actually produces relatively less potential change than the weaker shock in B*2*.

Katz states that the local response begins to appear as the stimulating shock reaches half threshold and that it then grows at an accelerating rate as the stimulus approaches threshold. If so, record B*2* from a half threshold shock, should be practically free of local response, while record B*1* should show the maximal local response. It is obvious, however, that there is no difference between them.

In figure 2C, a tracing showing a late diphasic spike recorded from the stimulated locus (heavy line) is superimposed on the tracing of the shock escape (broken line) obtained when the stimulus was reduced to

half threshold and the amplification doubled. The results obtained with this diphasic, and therefore conducted, response are in agreement with those obtained in 2, B.

Rushton (1937) has calculated that if the spike has three times the intensity necessary for propagation the local potential would be 13.5 per cent that of the spike. Preliminary experiments in this laboratory on anodally polarized nerve indicate that the normal spike probably has not even three times the intensity necessary for propagation. But accepting Rushton's value for purposes of discussion, the local potential from the fiber recording in A*2* and A*3* should cause B*1* to be 50 per cent higher than B*2*. Moreover, there are probably 20 fibers in the phalangeal nerve whose thresholds are less than twice that of the most excitable fiber. The threshold shock for the fiber under observation would therefore be expected to induce local responses in many others, or a total probably not less than five times that from the single fiber. On this basis one would expect B*1* to have four times the amplitude of B*2*. Rushton accepts an upper limit of ten times threshold as the stimulating power of the spike. Then B*1* should be about twice as high as B*2*. The method used here certainly is adequate to show variations of such magnitudes. It should be added that while the latter parts of the two records are essentially alike, there has been a consistent deviation in the earlier part, the weaker shock producing the higher record. This deviation may be due to distortion of the brief shock by the amplifier; since it seems to have no bearing on the immediate problem, the phenomenon has not been investigated.

It is obvious (*a*), that the sensitivity of the apparatus employed has been more than adequate to record any unconducted potential difference in nerve of the order described by Katz (1937, p. 269) or estimated by Rushton (1937, p. 239); (*b*), that the shock artifact is so small that it could not obscure a subthreshold response (Katz, l.c.); and (*c*), that failure to see such a response cannot be ascribed to the use of a device for balancing the escape, since none was used. The present experiments confirm previous experiments from this laboratory in failing to demonstrate any electrical phenomenon in nerve which does not vary directly with the subthreshold applied potential. There is not in the phalangeal preparation of the green frog a nonconducted electrical response with the characteristics described by Katz.

It still remains to account for the results recorded by Katz. In this connection reference may be made to two of his procedures that require clarification. (*a*) It seems that the lead arrangements were such that, were there a local nonconducted response of the same character as the conducted response, the negative variation should have disappeared, or at least decreased, when the local response reached propagating intensity

and imposed a diphasic voltage on his slow galvanometer, but this did not happen. (*b*) The rates of stimulation employed by Katz in these experiments are far in excess of the number of impulses the nerve can carry; the high frequency not only may have introduced the complication of Wedenski inhibition, but also involves the use of an excessive quantity of current for stimulation, with the possibility of magnification of artifacts such, for example, as rectification by the nerve.

It may be added here that Hodgkin (1937) has described, in crustacean nerve, a local potential at the lead that increases more rapidly than the stimulus voltage as the latter approaches the threshold of the conducted response. The phenomenon is particularly obvious when a shock is used which infrequently results in propagated responses. As the nerve's excitability varies spontaneously (the range probably is narrow) the potential at the lead shows a variation which amounts to about 35 per cent of the maximum.

Such a phenomenon is not seen in frog's nerve. The potential developing at the electrode in response to a shock which just fails to stimulate, is perfectly constant; there is no demonstrable variation, not even one of the order of the spontaneous variation in the excitability of the nerve fiber. In this connection figures 2 and 6 should be compared with the figure shown by Hodgkin (1937).

Now, it is possible, by means of anodal polarization, to so depress medullated nerve that an impulse initiated by an adequate shock will fail of propagation. But under such circumstances, records from the depressed locus show that when the imposed shock attains a critical voltage an all-or-nothing response of a single internodal segment occurs. Increases in shock strength then may result in the growth of the spike by all-or-nothing steps until the response extends beyond the depressed locus, when conduction proceeds normally. Similar results have been obtained, without polarization, from a damaged fiber which failed to conduct in spite of a relatively low threshold. A local response, then, can be demonstrated in medullated nerve when it is depressed to the extent that propagation does not occur. Such a local response, however, grows with increasing strength of stimulation in a quantal manner with entirely reproducible steps comparable to the steps seen in the quantal blocking of a conducted response (Erlanger and Blair, 1934), not in the smoothly graded fashion observed in crustacean nerves. Since the least demonstrable local response in a nonconducting medullated fiber represents the contribution from a segment, one favorably placed in relation to the lead, its contribution constitutes a significant portion of the spike record. Likewise in the experiments on normal nerve, any local response involving a segment stimulated at the lead would unquestionably have been observed had it occurred. It, therefore, follows that the least unit of a medullated fiber that responds

to a stimulus, the internodal segment, apparently is adequate for propagation of the nerve impulse. Possibly the myelin so modifies the path of the stimulating current that the whole segment is stimulated uniformly, and therefore fires as a unit, or else the segments are physiological units which respond to stimulation in an all-or-nothing manner. While we are inclined to favor the latter view, the information available at the present time does not warrant discussion of the possibilities.

Although there is no local response in normal medullated nerve, there is an "electrotonic" effect at the cathode which reaches a maximum long after the applied potential is removed, but this change is without a threshold, and, in the range studied, varies directly as the applied voltage.

II. *The relation of shock strength to latent addition.* Failing to find either the local physiological or the local electrical phenomena demanded by the hypothesis of partial response, it seemed advisable to examine the observation basic to that view, namely, that the latent addition does not change linearly with the strength of the conditioning shock. In the present experiments the conditioning induction shock (S_1) was set approximately 11 per cent below threshold and imposed on the thick part of the nerve through electrodes E_4 and E_5, and the effect upon the nerve's excitability with time was "tested" through the same electrodes by determining the strength of shock (S_2) required to reach the threshold at various intervals. The latent addition curve showing the change in threshold to S_2 is shown in figure 3, A (heavy line).

The strength of a shock can be varied either absolutely by changing the voltage or relatively by changing the threshold of the tissue. Through anodal polarization the nerve threshold was raised from 111 to 193; the unchanged absolute voltage of S_1, 89 per cent threshold at first, thus became 51 per cent threshold during anodal polarization. The latent addition under these conditions is shown as the light curve in figure 3A. In spite of the fact that anodal polarization increases the duration of the latent addition effect as usually determined (Blair, 1938), the same shock applied to the nerve in the less excitable state produces the shorter latent addition period. The crossing of the curves that occurs at about 0.4 msec. is without significance in this connection; it is due to the fact that the normal nerve, but not the anelectrotonic nerve, shows a considerable amount of accommodation which leaves in its wake a period of postcathodal depression. The greater absolute effect of S_1 in polarized nerve when the two dissimilar shocks are superimposed, may be due to a change in the strength-duration relations.

These curves are brought to the same ordinate value and plotted on semilogarithmic coördinates in figure 3B. Except for the fact that the latent addition effect of the relatively weak shock does not decline along a simple exponential curve, the results are entirely in agreement with those

of Katz. Other experiments in which the voltage of S_1 was varied, produced the same results and confirm the basic observation of Katz that changing the strength of the shock modifies its latent addition effect. Usually the later portions of the latent addition effect depart from the simple exponential relation; the only curves which were exponential over the entire range are those in which the late latent addition effect was opposed by postcathodal depression.

In order to study the change in the effect of a subthreshold shock as a function of its intensity, the relation of the strength of S_1 to that of S_2 required for stimulation has been determined with fixed intervals between the shocks. The results of a typical experiment of this kind, with a fixed separation of 200 μsec., are presented in figure 4A. Apparently the curve may be resolved into two straight lines with a transitional segment. This relation is clearly seen also when the total voltage ($S_1 + S_2$) is plotted against the voltage of S_1, as in figure 4B. A similar result is obtained from the data supplied by Katz in his figure 4 when they are similarly plotted. For instance, if one selects 0.5 K as the fixed separation of S_1 and S_2 the curves indicated by open circles in figure 5 result. In configuration they closely resemble those of our figure 4.

With a greater fixed interval the discontinuity shifts towards a higher voltage of S_1. An example is shown in figure 7. Comparable curves derived from Katz' data using a separation of 3 K are shown in figure 5 (dots). At this greater separation the two shocks appear to sum almost linearly over a much greater range of S_1 voltages.

Since the line through points D and F in figure 4A passes through the point S_2 = threshold, it apparently is an expression of stimulation by S_2 with the addition of an increment from S_1. The slope of the line indicates that this increment is about 77 per cent of S_1. Similarly the line through points I and K, passing through S_1 = threshold, indicates that S_1 stimulates by summation with about 62.5 per cent of S_2. The nonlinearity of the relation of voltage to latent addition seems, therefore, to be referable to a change from stimulation predominantly by S_1 when it is strong, the interval between shocks short, and S_2 weak, to stimulation largely by S_2 when it is strong and the effect of S_1 weak. The change from predominance of one shock to predominance of the other can account for the discontinuity which led Katz to assume the entrance and growth of a local nonconducted response.

In a previous paper, Blair and Erlanger (1936a) have shown by a lead from the locus of stimulation, with the shock artifact controlled by a balancing device, that a nerve responds to a brief induction shock with a variable latency amounting to about 300 μsec. In the course of the present experiments records have been secured from the site of stimulation without the complication of a balancing circuit, and without the compli-

cation of a slowly declining applied potential. The results of such an experiment are shown in figure 6. The delay in the appearance of the spike and the spontaneous variation in this latency are in complete agreement with results previously reported and confirm the conclusion formulated at that time, that excitation by a shock attains its maximum some 300 μsec. after the shock. Even when a very brief diphasic shock is used, such that the responding region is slightly anodal at the time of the response, a comparable variable delay is obtained (see fig. 2).

If we assume (1) that the temporal configuration of excitation produced by S_1 is identical with that produced by S_2 and (2) that the amount of excitation varies directly as the shock voltage, it is possible, with the information gained above, and that contained in figure 4A, to derive the approximate temporal configuration of excitation from a brief shock. S_1 attains its maximum at about 300 μsec. At that time about 62.5 per cent of S_2 is available for stimulation. Since S_2 is 200 μsec. later than S_1, shock excitation rises to about 62.5 per cent of its maximum in 100 μsec. At the time of the maximum effect of S_2, which is 500 μsec. after the start of S_1, the latter has fallen to about 77 per cent of its maximum value. A curve drawn through these three points should roughly reproduce the temporal configuration of the curve of excitation determined by a short shock. These curves for S_1 and S_2 are shown in figure 4C.

If these assumptions are valid, reconstructions made by summing excitations by S_1 and S_2 of the above configuration at a separation of 200 μsec., and of heights determined by the voltages read from curve 4A, should attain a threshold value for all points on that curve. The

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Fig. 3. A. Effect of the relative strength of a shock on its latent addition. Ordinates: voltage. Dots: latent addition curve when the threshold of the nerve is 111. Circles: latent addition curve from the same shock when the threshold has been elevated to 193 by anodal polarization.

B. The curves in A brought to the same ordinate values in semilogarithmic coördinates.

Fig. 4. A. Relation of the voltage of a conditioning shock (S_1) to that of the testing shock (S_2) required for stimulation. Interval between shocks, 0.2 msec.

B. Curve derived from the data in A, to show the relation of the total voltage required for stimulation ($S_1 + S_2$) to the voltage of the conditioning shock (S_1).

C. Derived curves of the temporal configuration of excitation determined by short shocks. Points, other than those for crest time, derived from A.

D to K. A family of curves showing how the excitations from two shocks with configuration shown in C, and voltages read from A, sum to attain threshold (upper horizontal line in each case). The curves labelled *D* to *K* portray the conditions obtaining at the correspondingly lettered points in A. Further explanation in text.

Fig. 5. Data obtained from figure 4 of Katz (1937) plotted as in figure 4 A above. A shock separation of 0.5 *K* seems to be comparable to the separation of 0.2 msec. obtaining in figure 4 A, and a separation of 3 *K* appears to be comparable to the data presented in our figure 7.

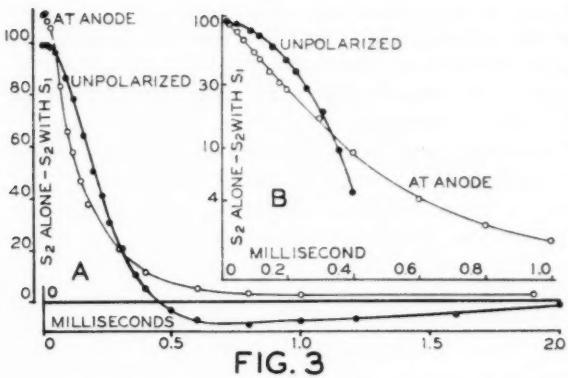

FIG. 3

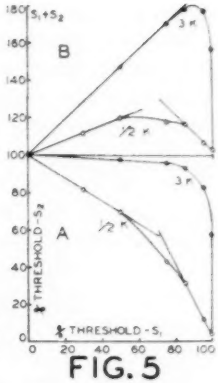

FIG. 5

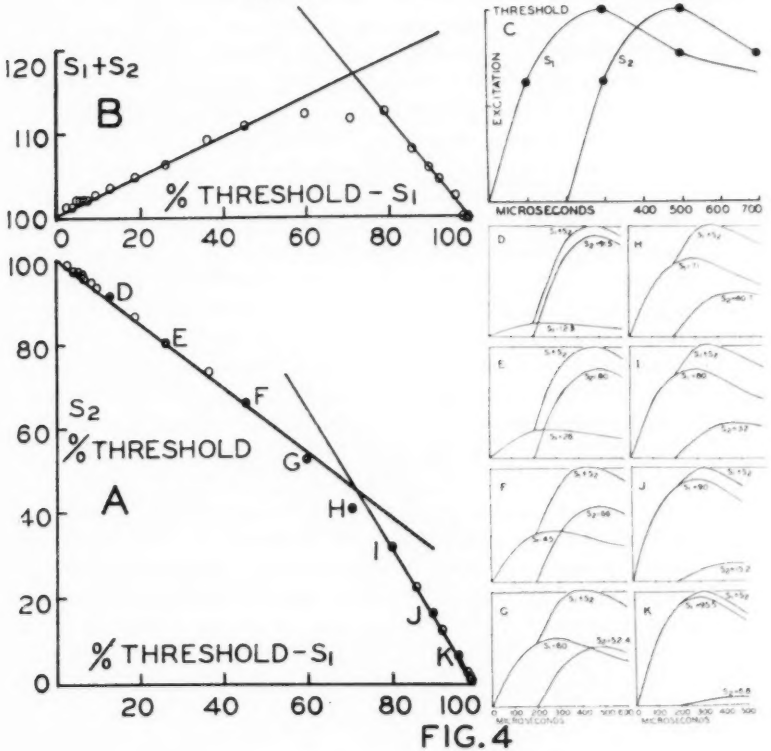

FIG. 4

family of curves, figure 4, D to K, thus derived all ascend to threshold (the upper enclosing line in each case) with a maximum error of 2 per cent. The derived curve of excitation therefore must closely simulate the actual curve of excitation by short shocks, and the summed curves the temporal configuration of excitation determined by the interaction of two short shocks of various voltages.

From the curves of figure 4, D to K, it is possible to make certain predictions. *a*. With a 10 per cent variation in threshold the time from the beginning of the shock to the development of the response should vary through a range slightly greater than minimum response time. *b*. When S_1 is strong the S_1 response time should be short; when it is weak and S_2 strong, this time should be longer.

Actually, a wide range of spontaneous play always is present; it complicates determinations of the time from a threshold shock to the appearance of the response. Consequently many determinations are required to reach any conclusion as to changes in shock-response time. Nevertheless, it can be shown by records from the stimulated locus that both the range of variation, and the average, of the S_1 response time change as the contributions from two temporally separated shocks are altered. The results of an experiment in which the phalangeal nerve (instead of the sciatic) was stimulated and the responses recorded from the locus of stimulation are shown in figure 7. As in figure 4, the fixed separation between the two shocks was 200 μsec. The upper curve, in which the total applied voltage ($S_1 + S_2$) is plotted against the voltage of S_1, demonstrates that the effectively linear relation discussed above holds through a wide range. The vertical lines in the lower graph express, for each voltage, the extreme range and the circles the average of the time from S_1 to the appearance of the response. Despite the wide range of spontaneous variation, it is clear that when S_1 is very near threshold the response time is much shorter than when S_2 is more largely responsible for stimulation. Data secured from the phalangeal nerve display the discontinuity of the linear relations of S_1 to S_2 at a higher voltage of S_1 than those obtained from the sciatic nerve, as may be seen by comparing figures 4 and 7. While the time to the maximum effect of the sum of two shocks separated temporally would not be expected to remain constant as their voltages are varied, actually, as has been seen, there is a range through which the variation is so slight that the summation to all appearances is linear.

To account for this result, one must assume that, in determinations of the latent addition effect of a shock that almost attains threshold, with short intervals between shocks, a portion of the rising excitation from S_2 suffices to raise S_1 to the threshold. The maximum of the sum of excitation then would occur effectively at the maximum of the excitation from S_1 and there should be relatively little change in the time from S_1 to the

appearance of the response. Since stimulation occurs before the maximum excitation from S_2 is attained, the time from S_2 until the appearance of the response would decrease as the separation between shocks increases. With wider separations, such that S_2 does not attain any significant fraction of its maximum excitation at the time of the maximum effect from S_1,

Fig. 6

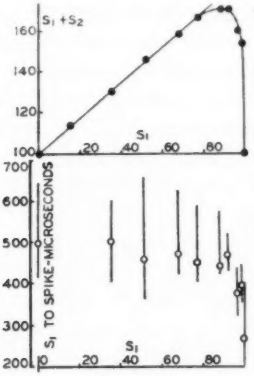

Fig. 7

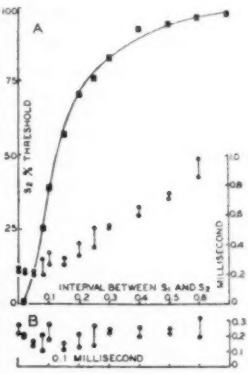

Fig. 8

Fig. 6. Four unretouched superimposed records obtained from the site of stimulation; shock (RC = 25 μsec.) just threshold. Time 10,000 cycles. There is not the slightest evidence of a spike in the lowest record ("escape" only); it serves as a base line for measurement of the spikes. The starts of the spikes play about the second crest of the shock "escape".

Fig. 7. Phalangeal nerve stimulated at the lead. Fixed interval between shocks 0.2 msec.

Upper curve: relation of S_1 expressed in per cent of threshold to the total voltage required for stimulation.

Lower curve: abscissae as above. Ordinates: time from the beginning of S_1 to the start of the spike at the cathode. Circles indicate the average shock-response time; the vertical lines, the range of spontaneous play. In the range in which the upper curve becomes nonlinear there is a significant change in the time of the appearance of the response.

Fig. 8. A. Latent addition curve of a shock 0.99 threshold (squares); dots, ordinates on the right, indicate the time from S_1 to the response. The separations of connected points indicate the range of determinations.

B. Range of the time from S_2 to the response. Abscissae as in A.

the S_1 spike time should vary practically linearly with the separation of the two shocks, and the S_2 spike time should return to a constant value. These predictions are verified by the data collected in figure 8. It may be seen that in the range in which S_1 is largely responsible for stimulation (something less than 0.2 msec. under these conditions) there is little change in the time from S_1 to the appearance of the response; the time from S_2

to the response decreases. As S_2 begins to predominate, the S_1 response time increases linearly with the separation while the S_2 response time becomes constant.

The curves in figures 7 and 8, the data for which were secured by stimulation of the phalangeal nerve at the lead, indicate that the period during which S_1 predominated as the stimulus was short; indeed the curve in figure 8, with the conditioning shock 99 per cent threshold, is very nearly exponential. We have been unable to secure from the very small phalangeal nerves curves matching figure 4. When S_1 and S_2 are separated by 200 μsec. and determinations made of the relative strengths required for stimulation, it is found that at the time S_2 stimulates there is, in the case of the sciatic, a residuum of about 77 per cent of excitation from S_1, in the phalangeal nerve only about 13 per cent.

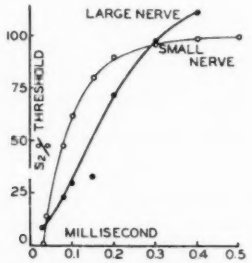

Fig. 9. Latent addition curves from the same fiber at two loci. Dots, fiber stimulated in the sciatic nerve; circles, in the phalangeal nerve. The latent addition effect is much more pronounced at the sciatic locus in spite of the fact that accommodation is much more evident there.

This difference led us to suspect that latent addition might be influenced by inactive tissue surrounding the nerve fiber. A preliminary experiment, planned to ascertain whether this is the case, has shown (fig. 9) that one and the same fiber may have a very much longer latent addition period when stimulated in the sciatic nerve, than when it is stimulated in the phalangeal nerve. The crossing of the curves (in the vicinity of 0.3 msec.) is referable to the fact that in the large nerve there was pronounced postcathodal depression, in the small nerve, very little. Blair and Erlanger in previous papers (1936a, 1936b) have ascribed the local excitatory process, and therefore, latent addition effects, to a distortion of the applied potential by the complex electrical circuit of the nerve. It is not surprising, therefore, that the amount of tissue surrounding the fiber influences the configuration of latent addition curves.

If there is a redistribution of the stimulating current through the nerve such that the voltage at the responding locus reaches a maximum some

300 μsec. after the applied potential is removed, it should be possible to see something of it in records from the stimulated locus. As a matter of fact records of the response to just subthreshold shocks do show a second elevation around which the beginning of the response varies (figs. 2 and 6). The temporal configuration of this "shock escape," or electrotonic potential, is independent of the voltage of the subthreshold shock and its height varies directly as the applied potential. The peak of the electrotonic voltage produced by a threshold shock with an RC of 25 μsec. is of the same amplitude as that produced by a threshold shock with an RC of 4.3 μsec. Their general configurations also are very similar. This behavior of the "shock escape" is common with that of the "local excitatory process." There are, however, two important differences between them. *a*. As shown by figure 4, D to K, stimulation should occur when the sum of the two shock excitations is equal to threshold. But though the shock escape amplitude is the same for different just threshold shocks, it is higher when two shocks interact to stimulate. *b*. The temporal configuration of the shock escape is similar to, but not identical with, the local excitatory process as determined from latent addition curves.

Since the resistance of the phalangeal nerve is high, and therefore materially slows the input stage of the amplifier, it is possible that these discrepancies are due to amplifier distortion of the short shock with the consequent addition of a significant element to the record of the electrotonus. Therefore, though the results are suggestive, the question as to whether there is a change in excitability of nerve paralleling the temporal configuration of the electrotonic potential will have to be left open pending studies in which the direct effects of the applied voltage can be better controlled.

I wish to express my indebtedness to Dr. Joseph Erlanger for his valuable suggestions during the course of the experimental work included in this paper, for assistance in the analysis of the material, and for his help in this presentation of the results.

SUMMARY

Observations have been made to test the existence in normal, medullated frog's nerve of a local nonconducted response to subthreshold shocks. The experiments include records of spikes of single axons obtained from the stimulating cathode without the use of any balancing device. The following results have been obtained.

1. Subthreshold shocks produce no refractoriness and neither block nor slow conduction.

2. The only observable local negativity produced by a subthreshold shock varies directly as the applied voltage.

3. When a conditioning shock is just subthreshold and is followed after a brief interval by a weak testing shock, stimulation occurs early, very nearly at the time of maximum excitation from the conditioning shock. When the conditioning shock is weak, or the separation between the two shocks wide, the testing shock predominates and the response occurs at a later time. This change in the time of the response is responsible for the inconstant relation of conditioning shock to latent addition.

4. From latent addition curves information may be obtained as to the temporal configuration of the "local excitatory process." It confirms the views on nerve stimulation by short shocks previously advanced by Blair and Erlanger (1936a).

5. The intensity of the "local excitatory process" appears to vary linearly with the voltage of a subthreshold shock.

6. The temporal configuration of the "local excitatory process" appears to depend in part on the amount of shunting tissue.

7. There are indications that the excitation from a shock (local excitatory process) has the configuration of the electrotonic potential.

8. In depressed nonconducting medullated nerve, the all-or-nothing response of a single segment is the least obtainable; increasing the strength of stimulation increases the spike through the entrance of additional segment responses.

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THE PROGESTIN CONTENT OF THE CORPUS LUTEUM OF THE SOW (SUS SCROFA) DURING SUCCESSIVE STAGES OF THE ESTROUS CYCLE AND PREGNANCY

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In order to understand better the functional rôle of the corpus luteum, we have undertaken the quantitative determination of the progestin content of the sow's corpus luteum during successive stages of the estrous cycle and of pregnancy. In spite of the obvious desirability of such information the difficulties of collecting sufficient dated material for assay are so great that up to the present there is only one brief report, that of Elden (1934; see below, p. 475).

Our material was collected from the abattoir, and was sorted into lots representing the various stages of the cycle and of pregnancy by a necessarily tedious method. Reproductive organs of sows from the slaughterhouse may be dated by application of Corner's (1921) description of the histological changes in the ovaries and uterus. The estrous cycle is typically of 21 days' duration. The first three days of the cycle, beginning with the day of ovulation, may be recognized by the presence of recently ruptured follicles in the ovary and ova in the fallopian tubes (discovered by washing out the tubes with salt solution and examining the washings with a dissecting microscope). The period from the 3rd to the 15th days, during which the corpora lutea attain and hold the structure of active endocrine tissue, may be divided into two parts by examining the epithelium of the uterus. After the 15th day the corpora lutea are visibly retrogressive; in case of doubt a section of the corpus luteum may be made.

From each pair of ovaries the corpora lutea were dissected, weighed, counted, minced, and preserved separately in 95 per cent ethyl alcohol until the dating was ascertained, after which the corpus luteum tissue was added to the accumulated stock of its particular stage.

To secure the earlier stages of pregnancy, before visible enlargement of the uterus occurs, all uteri associated with ovaries containing mature corpora lutea were opened and if early embryos were recovered they were examined under the dissecting microscope and compared with the figures of Keibel's Normentafel (1897) to ascertain their age. In the absence of

pregnancy such uteri were dated by histological examination of the endometrium as described above. On the basis of Corner's description and of general experience, it was assumed that the corpora lutea of the first 15 days after ovulation are alike, whether or not the animal is pregnant. Ovaries from pregnant sows containing embryos less than 15 days were therefore added to those of the non-pregnant animals of similar stage. Thus group C (10th to 15th day after ovulation) contains both non-pregnant and pregnant animals, the former predominating. Group A contains no pregnant animals, because boars are seldom present in the herds destined for slaughter during the last few days before killing.

For the purposes of our study, pregnancy (beyond the 15th day) was divided into six stages, chosen on the basis of Corner's (1915) description of histological changes in the corpora lutea during pregnancy. Classification was determined by the crown-rump length of the fetuses.

Since for unknown reasons sows in the last 10 days of pregnancy, bearing fetuses from 220 to 290 mm. C.R.L., are very scarce at the Rochester Packing Company's abattoir, Dr. W. R. Lyons of the Department of Anatomy of the University of California was kind enough to collect for us at San Francisco 50 grams of corpora lutea from this stage. The method of collection and preservation was the same as that used for the rest of the material.

In the case of uteri associated with retrogressing corpora lutea it is obviously impossible to discriminate (in the absence of individual records) between those which are retrogressing at the end of a cycle and those retrogressing after parturition and abortion. Our material however almost certainly consists chiefly of specimens from the non-pregnant cycle. In order to exclude late stages of retrogression no such corpora were collected below 4 mm. diameter, the size to which they have diminished at the beginning of the next cycle.

The manner in which the material was classified into separate stages is shown in the first two columns of table 1.

The total weight of corpus luteum tissue collected from each stage is shown in table 1. With the exception of group J, for which only 50 grams could be obtained, the amount varied from 117 to 250 grams.

The tissue was extracted by the method developed in this laboratory and described by Allen (1930). To avoid loss of hormone, extraction was carried only to the stage of the crude oil.

The extracts were assayed on adult rabbits by the method described by Allen (loc. cit.). Because the amounts of tissue available were necessarily limited, our results are approximations rather than exact measurements. Their reliability is however greater than may at first appear, because we have been able to avail ourselves of certain experience acquired in this laboratory by which the assay may be made with the smallest

TABLE 1

	STAGE	NUMBER PAIRS OF OVARIES	TOTAL WEIGHT CORPORA LUTEA	AVERAGE WEIGHT OF SINGLE CORPUS LUTEUM	AVERAGE NUMBER CORPORA PER SOW	AVERAGE NUMBER GRAMS CORPUS LUTEUM PER SOW	AMOUNT EXTRACT INJECTED, .85 GRAM EQUIVALENT*	REACTION	GRAMS PER RABBIT UNIT†	YIELD IN RABBIT UNITS PER KGM.	YIELD IN RABBIT UNITS PER SOW
			grams								
Estrous cycle	A. 1st-3d day, ova in oviduct	87	103.5	0.113	10.5	1.19	44.6 58.5	++ +++	60	16.6	0.01
		104	122.8	Not obtained		1.18	60 62.6	++ ++	90	11.1	0.012
	B. 3d-10th day, ova degenerated	34	147.3	0.367	11.8	4.22	18 37 55	0 ++ +++	55	18	0.076
	C. 10th-15th day, either degenerate ova, or early embryos present	28	127.3	0.416	10.9	4.55	10 21 31	0 0 ++−+++	30	33.3	0.152
	D. 15th-20th day, embryos up to 20 mm.	27	127.3	0.429	11.0	4.72	10 20 45	0−+ ++++ ++++	20	50	0.235
	E. 20th-40th day, embryos 20-55 mm.	24	120.0	0.406	12.3	5.00	10 20 45	++ +++ ++++	20	50	0.250
Pregnancy	F. 40th-75th day, fetuses 55-140 mm.	32	142.1	0.450	9.8	4.43	15 20	+ +++	20	50	0.222
	G. 75th-105th day, fetuses 140-170 mm.	29	117.5	0.423	9.6	4.10	3.75 9.5 15.0 30.0	0 + +++ +++	15	66.6	0.270
	H. 105th-110th day, fetuses 170-220 mm.	41	176.3	0.410	10.25	4.31	15 15 25	++ ++ +++	25	40	0.176
	I. 110th-120th day, fetuses 220-290 mm.	15	54.5	0.491	7.4	3.63	54.5	0	>108	<9.25	<0.034
Retrogression of corpora lutea	J. 16th-21st day of cycle or equivalent stage of retrogression if animal had been pregnant	206	252.3			1.22	60 185	0 0	>370	<2.7	<0.003

* The figure expresses the number of grams of fresh corpus luteum tissue represented by the concentrate injected.

† The figure expresses the number of grams of fresh corpus luteum tissue found to contain 1 rabbit unit (i.e., it is the interpretation of the previous two columns).

possible number of trials. A progestational reaction of +++ or ++++ is by definition indicative of 1 rabbit unit. Experience shows that one-half to three-fourths of the minimal effective dose will give a ++ reaction. When, therefore, a reaction of +++ or ++++ is found, the dose administered is known to have contained at least one rabbit unit. One half the dose is then administered, and so on until a + or ++ reaction only is obtained. If the first trial gives less than +++, the dose is raised as suggested by the reaction actually obtained. If no progestational reaction is obtained, the dosage must be at least doubled to get a +++ reaction. In this way the minimal effective dose may often be ascertained with only two or three rabbits, especially if the investigator is able by reason of experience or judgment to hit upon an approximately correct dose at the start. Such assays are found to yield reasonably consistent results when repeated (cf. Young, 1936). One rabbit unit thus defined is approximately equivalent to one international unit (1 mgm.) of progesterone.

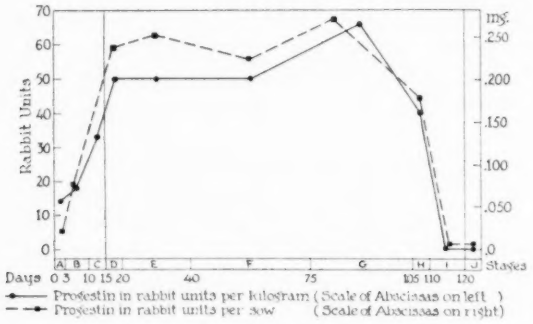

Fig. 1

As a check upon our results, it may be pointed out that our average yield from groups A to H inclusive (comprising all the stages present in the massed slaughter-house material used in this laboratory for routine preparation of progesterone) is 40 rabbit units per kilogram, a figure closely similar to yields obtained by W. M. Allen, the most experienced worker in this field.

Table 1 exhibits the results of the investigation.

In figure 1 the yields during the estrous cycle and pregnancy are plotted graphically against the time included in each period studied. The results are expressed both as rabbit units per gram of raw corpus luteum and as rabbit units per sow; the two curves are practically parallel. It will be seen that the secretion of progestin begins during the first days of the life of the corpus, reaching average yield of 16 rabbit units per kilogram during the first 3 days. At this time the endocrine cells of the corpora lutea are

not yet fully differentiated. There is a further slight rise in the progestin content in our second phase (3rd to 10th day). In this phase, as will be seen from the table, the weight of the individual corpus almost attains its maximum, while the maximum progestin content per unit of weight is still relatively low.

In the period of the 10th to the 15th day, during which in the event of pregnancy the embryos become attached to the uterine wall, there is a sharp increase of progestin content to 33 rabbit units per kilogram.

Figure 1 shows that during pregnancy there is a further rise in progestin content during the period from the 15th to the 20th day, followed by a period of high content lasting throughout the greater part of the gestation period. Our assays indicate, indeed, a further increase between the 75th and 105th day. Material collected after the 105th day shows a marked decrease, and during the last 10 days of pregnancy no evidence of progestin is obtained. After regression the corpora lutea contain no progestin.

The general conclusion from these results is that progestin is available, and therefore probably is required during all but the last few days of pregnancy in the sow.

It is possibly significant that the curve of progestin content during pregnancy is similar to the curve of combined cholesterol reported by Bloor, Okey and Corner (1930). The close chemical relationship of progesterone and cholesterol is now well known.

Elden's study (1934) to which reference has been made, was based upon the assay of four groups of sows' corpora lutea, as follows: 1, early corpora lutea from ovulation to the 6th or 7th day; 2, fully formed corpora representing the various stages of early and middle pregnancy; 3, degenerating corpora lutea; 4, corpora albicantia. He obtained his highest yields from the first group, lower yields in the second, and negligible yields in the retrogressive corpora. Compared with our results, Elden reported higher yields in the first week, but lower during pregnancy.

One statistical point requires mention, namely, the low number of corpora per sow in group I (late pregnancy) which had only 7.4 corpora as against averages of 9.6 to 12.3 in all other groups. Since our material of group I was that collected in California by Doctor Lyons, the discrepancy might have been ascribed to differences in breed or in ovulation rate between the swine marketed in San Francisco and in Rochester, were it not for the fact that an exactly similar difference was noted by Corner (1923) in swine all of which were studied in one abattoir in New York City. Possible explanations are discussed in Corner's paper; but the work of Wishart and Hammond (1933) and Hammond (1934) suggests a more plausible conjecture. They showed that in rabbits large litters are born earlier than smaller litters. If this occurs in swine it would lower the average litter-size, and consequently the corpus luteum count, in late pregnancy.

SUMMARY

1. The corpora lutea contain appreciable amounts of progestin during the first three days after ovulation, before the corpus luteum cells are fully differentiated.

2. During the estrous cycle the content rises until retrogression sets in at the 15th day, after which progestin cannot be detected.

3. During pregnancy there is a further sharp rise until the 20th day. The progestin content remains high and apparently rises, even into the fourth month. A drop occurs some time toward the 105th day, and during the last 10 days (110–120th day) progestin is not found in the corpora lutea.

The authors wish to express their appreciation to Dr. George W. Corner and Dr. Willard M. Allen for their advice and support in this study. Mr. Frederick Kesel gave valuable assistance in the collection of the material. Thanks are due to the Rochester Packing Company for facilitating the collection.

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VARIATIONS IN THRESHOLD OF AUDITORY STIMULI NECESSARY TO AWAKEN THE SLEEPER

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In a previous publication (1) we demonstrated that the classical depth of sleep curves do not give a true picture of the changes in irritability to external stimuli in the course of a night's sleep. Our studies showed that, instead of one continuous curve of the depth of sleep, there are many successive contiguous curves, with the low points between adjacent curves usually associated with motility of the sleeper, and the curves becoming briefer and shallower as the night progresses. However, we rarely tested the depth of sleep very soon after the assumed onset of sleep, as the subject and observer were in different rooms, and it was impossible to determine with accuracy just when sleep began. In the present study it was our purpose to plot this initial depth of sleep curve from points representing the thresholds of auditory stimuli necessary to awaken the sleeper during the first hour following the *visible* onset of sleep.

METHOD. As in our previous study the external stimulus used was a sound produced by a magnetic loud-speaker actuated by an electrical current from a 110 volt, 60 cycle A. C. outlet. The intensity of the sound was varied by the use of a rheostat, the dial of which was graduated from 0 to 100, with the voltage thus represented having been determined by the use of a voltmeter. When stimulating the subject the experimenter would turn the rheostat up to 10 and let that sound continue for 10 seconds. If the subject did not respond by saying, "I hear it," the sound was turned off for 10 seconds, following which the rheostat was then turned up to 20 and held for another 10 seconds. If this did not awaken the subject, the sound was again turned off and then turned on again after a 10-second interval at 30; and so on, until the subject awakened enough to respond by the pre-arranged signal, or until the maximum of obtainable loudness was reached. The normal wakeful level of irritability was determined before each sleep test. The sound stimuli were given at various time intervals after the onset of sleep, which in the case of the adults was determined by having the subject hold a paper loosely in his hand; when the

[1] Aided by a grant from the Rockefeller Foundation.

paper dropped from his grasp, he was judged to be "going to sleep." In every case other criteria (such as changes in respiration, muscle twitchings, etc.) were also used in aiding the experimenter to decide as to the time of the onset of sleep. In doubtful cases the subject was questioned as to whether he was or had been asleep.

Since we were interested only in the irritability changes associated with the initial period of sleep, most of these results were obtained on subjects taking an afternoon nap or an early evening nap, though some were on subjects going to sleep for the night at their regular bedtime. The experimenter observed the subject continuously throughout each testing period, which lasted up to an hour and a half. During this period the subject was stimulated from one to half-a-dozen times.

RESULTS. Over two thousand individual threshold determinations were made on twenty sleepers divisible into three groups: eight normal adults, five normal children from 4 to 7 years of age, and seven feeble-minded adults with I.Q. ratings of 30 to 50. The composite threshold of irritability curves (of which the depth of sleep curves would presumably be vertical mirror-images) for these three groups are superimposed on each other in figure 1. They represent variations in irritability to sound during the first sixty minutes of sleep. They resemble each other strikingly and can each be divided into three parts: 1, a period of gradually rising thresholds lasting about 25 minutes and showing distinctly first a positive, then a negative acceleration, or a typical S shape; 2, a period of practically unchanged irritability, or plateau, of about 25 minutes' duration; and 3, a ten minute period of rapidly decreasing thresholds approaching but not reaching the waking irritability levels.

In individual cases the curves differed not only with respect to both duration and height, but also as to the distribution of the three periods. Some individuals habitually reached the plateau sooner than others. The same individual showed a more abbreviated than usual first phase and a more retarded beginning of the third phase when he was very sleepy. In practically all cases the low point of the descent of the curve was associated with stirring or other evidences of lighter sleep.

Response to stimulation did not materially affect the course of the curve. It appears that the sleeper may wake enough to respond to the sound and yet not be fully awake, as he failed to return to the waking level of irritability and usually went back to sleep at once, without stirring. Thus, if the subject was awakened by the stimulus within a few minutes after first dropping off to sleep and then allowed to go back to sleep, and again stimulated soon thereafter, and this procedure repeated several times in succession, it was found that the threshold of irritability gradually increased with each successive test.

There were also group differences in the variations of the auditory

threshold. The waking thresholds were lowest for the normal adults, somewhat higher for the children, and still higher for the feeble-minded. The first portions of the three curves are more completely superimposable than the others, the feeble-minded group, however, showing consistently higher thresholds than the normal two groups. In the plateau portions of the curves, the children show an upward slant, the feeble-minded a somewhat downward direction, with the normal adults more nearly horizontal. In the descending portions of the curves, the threshold of the children does not drop nearly as low as that of the adults.

The individual curves of the normal adults were more nearly alike than those of the children and the feeble-minded. However, the differences

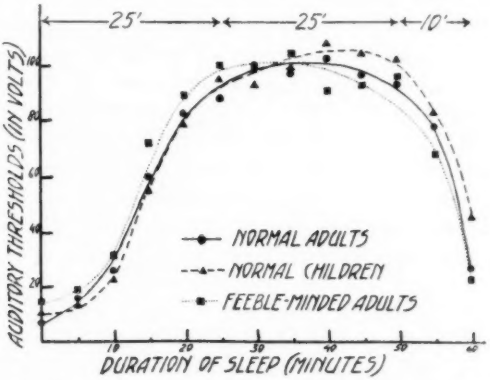

Fig. 1. Superimposed curves of auditory irritability changes during the first hour of sleep in three groups of subjects. For the normal adults the plotted points are based on the averages of 996 determinations on eight subjects; for the children, on 571 determinations on five subjects; and for the feeble-minded, on 668 determinations on seven subjects—a total of 2,235 trials on 20 individuals. The higher the threshold, the greater the depth of sleep.

in all cases were not such as to affect the general shape of the irritability curve, or those of its constituent parts.

Discussion. There is a striking similarity between the curve of the variations in the threshold of auditory stimuli necessary to awaken the subjects during the first hour of sleep and two curves previously reported by us (1): *a*, the curve of irritability changes in dogs at the onset of sleep, and *b*, irritability changes in human subjects during the entire night in relation to the time that elapsed after a spontaneous movement of the sleeper. They undoubtedly all represent the same phenomenon—a gradual development of a certain depth of sleep which is then maintained for a longer or shorter period of time. In the case of dogs we find that the S-shaped curve at the onset of sleep requires 30–35 minutes for its

development, instead of 25, thus resembling the children's curve more than those of the adults. The human successive contiguous curves previously studied by us, on the contrary, showed an S-shaped curve of shorter duration, 15 to 20 minutes, and the plateau was not so high, with an average of about 70 volts, compared to about 100 for the initial curve here reported. This indicates that not only do the contiguous curves get irregularly shorter and shallower, but also that the first curve is the longest and deepest. With the additional information, we can thus draw a schematic "typical" depth of sleep curve, or, rather, a series of curves for the whole night, all of them having the general allure of the initial curve (fig. 1), but which toward the end of the sleep period are only about one-fifth as long and as deep as this initial curve.

It will be recalled that we counted the "onset" of sleep from the moment the subject would drop a piece of paper held between two fingers, which indicated that he had reached a certain degree of muscular relaxation. But the fact that the irritability to sound stimuli progressively decreases for a period varying from a few minutes to half an hour, raises the question as to whether there is not a *period* of onset of sleep rather than a *moment*. Davis, Davis, Loomis, Harvey, and Hobart (2) came to a similar conclusion on the basis of the number of different brain wave patterns they could discern in the human subject during the passage from drowsiness to deep sleep.

Concerning the S-shape of the curve, we find similar curves in the development of conditioned reflexes and in curves of learning. Various hypotheses have been proposed to explain these latter, among others, that we are dealing here with a mono-molecular autocatalytic reaction. Whatever the true explanation of any of these, it is interesting to note the similarity between the establishment of the state of sleep and that of other functional states of the central nervous system.

The fact that the curves for children, adults, and the feeble-minded are so closely alike indicates that neither physical nor intellectual development modifies the process of the development of the state of sleep.

Lastly, the failure of the curve to start *de novo* after the sleeper had responded to stimulation suggests that he was not fully awake at the time, and this is supported by his frequently not being able to recall whether he responded or not. It suggests, furthermore, that it is not necessary for an individual to be fully awake in order to perform simple acts, and we undoubtedly go through a number of such semi-waking states during an ordinary night's sleep.

SUMMARY

On the basis of variations in the threshold of auditory stimuli necessary to awaken the sleeper, it was demonstrated on twenty subjects (normal

adults, normal children, and feeble-minded adults) that following the onset of sleep there is, first, a gradual S-shaped decrease in irritability reaching a maximum in about 25 minutes; second, a period of sustained low irritability of similar duration; and third, a fairly rapid increase in irritability, not quite to the waking level, during the next ten minutes. The whole duration of the initial depth of sleep curve is about one hour, and it is usually not duplicated either in length or in depth during the rest of the night's sleep.

We were kindly granted the privilege of studying the inmates of the Lincoln State School and Colony at Lincoln, Illinois, during the summer of 1937, and we wish to express our thanks to the Managing Officer of the School, Dr. F. A. Causey, and to the members of his staff, for the help they gave us during the work at their institution.

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DIRECT EVIDENCE OF FUNCTION IN KIDNEY OF AN EARLY HUMAN FETUS[1]

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The literature on function of the early fetal kidney, beginning with the first report in 1827 and following it until 1930, has been well reviewed by Needham (1). He describes the findings of various authors who report the presence of urea and uric acid in the amniotic liquid and, in the later stages of gestation, in the ureters. One of the more recent investigations is that of Guthmann and May (2) who reported the finding of urea in the amniotic liquid of human embryos beginning at $2\frac{1}{2}$ months and its increase with the age of the embryo. The authors therefore inferred that the human embryonic kidney begins at $2\frac{1}{2}$ months to function. A number of workers have reached the same conclusions by histological methods, e.g., Hewer (3) found that at the 12th week of gestation there was a differential staining of proximal and distal tubules which was absent in kidneys of earlier age. From this she concluded "that the human fetal kidney is certainly functioning as early as the 12th week and probably as early as the 9th week." Gersh (4), in a recent comprehensive paper on the correlation of function and structure in the meso and the metanephros claims, from purely morphological data, that tubular function begins in the human embryo at about 9 weeks.

We were fortunate in obtaining a human fetus immediately after delivery by Caesarian section, the estimated age of which was $3\frac{1}{2}$ months. An opportunity was thus presented for repeating on the human metanephros the previous experiments on the accumulation of phenol red by the mesonephric tubules of the chick in tissue culture.

Recently the tissue culture method has been extensively applied (5, 6) to a study of different phases of renal activity of the chick mesonephros by observing the accumulation, under controlled conditions, of various dyestuffs in the lumina of the tubules. Phenol red (phenol sulfon phthalein) and Orange G (C. I. No. 27) in aqueous solutions are examples of dyes which are not typical vital stains but which are accumulated by a

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process of secretion by certain specialized cells undergoing metabolic activity (7). During this process the cells become colored.

The kidney of the human fetus was removed aseptically, cut into pieces and small fragments teased out from the cortex in sterile Tyrode solution. The fragments were embedded on coverslips in a medium consisting of 1 part chick plasma, 3 parts human serum, 1 part human embryo extract prepared from the remainder of the same embryo, and sealed over depression slides with an air space of about 2.3 cc. The explants included glomeruli, segments of proximal tubules identified by their high columnar epithelium, loops of Henle, distal tubules and collecting ducts. The cut ends of the tubular segments heal over and the secretory activity of the closed segments is determined by the accumulation of fluid in and subsequent distention of their lumina. Attention was given to the tubules in the explant and not to eventual unorganized outgrowths.

Phenol red was mixed with the medium of some of the preparations and Orange G with that of others. These two dyes were selected because they are practically non-toxic. In our work on the chick mesonephros (8), we found that phenol red rapidly penetrates the cells of the proximal tubule and is secreted by them into the tubular lumina of the isolated segments. The dye, as a result, accumulates in concentrations far above that of the medium. Orange G may also enter the lumina in this manner or it may be segregated into vacuoles in the cells.

Cultures with phenol red. Phenol red in aqueous solution is an indicator having a pH color range from yellow at 6.8 to red at 8.2. The teased out fragments of the human metanephros were placed in the medium as already described and one drop of phenol red (0.5 per cent solution) added to 1 cc. of the medium (final concentration of about 0.025 per cent). This mixture assumed the yellow-pink color indicative of pH 7.4 to 7.6.

Examination of the cultures after three hours of incubation showed that the ends of the tubular segments had healed over and that the walls of about 40 per cent of the proximal tubules showed a diffuse pink-yellow color (cf. 9). The remainder of the tubules in the cultures were uncolored, including all the loops of Henle, distal tubules and collecting ducts. The renal corpuscles were also colorless which was to be expected because of a complete lack of blood circulation. The dye reaches the cells of the tubules by diffusion through the medium. Occasionally, a small amount of the dye was seen within a torn glomerular capsule but the color intensity was never greater than that in the surrounding medium. The lack of coloration in some of the proximal tubules is a condition frequently met with in tissue cultures of kidney fragments.

After 24 hours of incubation, the cultures contained the same proportion of colorless tubules as at 3 hours' incubation. The phenol red in the medium retained its original color while the colored tubules, all of which

were proximal, now showed yellow walls and some distention with an accumulation of phenol red as a yellow liquid in their lumina. At 48 hours the intensity of yellow color in the lumina indicated a concentration of an aqueous solution of 0.05 per cent progressively increasing while that of the medium lessened appreciably. The yellow color of the phenol red in the lumina indicated that the tubular urine was acid, with a pH of 7.0 or less, although the medium retained the color of the alkaline range, indicating a pH of 7.4–7.6. The relatively acid reaction of the human proximal tubular fluid is in marked contrast to that which obtains in the chick mesonephric tubules. As already mentioned the kidney cultures were mounted on large depression slides and thus exposed to a considerable amount of air. Under these conditions the lumina of the proximal tubules of the chick have invariably indicated the same pH as that of the medium although the concentration of the dye in the lumina was markedly higher. If cultures of chick tubules are covered by a depression slide with a much smaller air space (approximately 0.5 cc.), the red color in the lumina and in the medium changes, after 10 to 12 hours, to the acid, yellow color, indicating a pH of 7.0. This change is presumably caused by an accumulation of CO_2 in the air chamber, since, when the culture is opened and exposed to air, the color in the medium and in the lumina returns to red in a few minutes. On the other hand, the relatively acid state of fluid in the lumina of human proximal tubules is not appreciably affected by exposure of the cultures to air. Although the medium changes immediately in correspondence to the loss of CO_2, the acid reaction of the fluid in the lumina remains unchanged as evidenced by the persistence of the yellow color.

Cultures with Orange G. Orange G is yellow in aqueous solution and has no pH color change. Cultures were prepared and one drop of Orange G (0.05 per cent solution) added to 1 cc. of the medium making a final concentration of about 0.0025 per cent. The Orange G solution was used in a weaker concentration than that of phenol red. Orange G at 0.025 per cent gave too intense a color for observation and tended to be slightly toxic. The preparations showed progressive accumulation of yellow fluid in many of the tubular segments, the walls of which became colorless while the lumina became increasingly distended. As in the cultures with phenol red, glomeruli, loops of Henle, distal tubules, and collecting ducts remained colorless. After 72 hours a majority of the proximal tubular segments had developed into large balloon-like cysts containing yellow fluid which was much deeper in color than the surrounding medium. The concentration within the lumina gave a color intensity equal to an aqueous solution of about 0.01 per cent Orange G, while the medium immediately surrounding the explant, lost color to less than 0.001 per cent. This one-way passage of dye and fluid into the lumina with no

means of elimination, also occurring in tubules with phenol red, was even more strikingly demonstrated in the cultures with Orange G. The colorless walls in these preparations showed up sharply against the brilliant yellow distended lumina while with phenol red both walls and lumina were yellow and the lumina showed far less distention. No yellow vacuoles appeared in the walls of any of the tubules. The lack of vacuole formation also occurs in some of the tubules of the chick mesonephros where, however, the intracellular vacuole formation predominates. It is suggested that this readiness of Orange G to pass through the wall without becoming segregated in cytoplasmic vacuoles indicates a very active state of tubular function.

SUMMARY

1. The cut ends of segments of the tubules of a $3\frac{1}{2}$ month human embryo kidney heal over in tissue culture.

2. Phenol red (phenol sulfon phthalein) and Orange G (C. I. no. 27) if present in the surrounding medium, pass into and accumulate in the lumina of the proximal tubules to a conspicuously higher concentration than that of the dyes in the medium. In this regard the human metanephros behaves, essentially, in the same manner as the chick mesonephros.

3. The experiments with phenol red show that the proximal tubular fluid of the human metanephros has a pH of approximately 7.0 while the culture medium is from 7.4 to 7.6. This is in marked contrast to the conditions in chick mesonephros in which the pH of the proximal tubular urine approximates that of the surrounding medium which is 7.8.

4. These results constitute the first direct evidence that human embryonic proximal tubules of themselves are able, at least at $3\frac{1}{2}$ months, to function.

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HEAT EXCHANGES OF MAN IN THE DESERT

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The measurements of heat exchanges presented here were planned in order to ascertain the rates at which heat was gained and lost, both at rest and during exercise, in dry hot atmospheres. Human endurance in hot climates might possibly be limited by the rates of heat dissipation, great accumulations of heat in the body being thus prevented.

This study is a part of the work carried out by the Harvard Desert Expedition at Boulder City, Nevada, in the summer of 1937. The seven members of the expedition served both as subjects and observers.[1] A previous expedition had been made by some members of the party to the same place in the summer of 1932 (Talbott and Michelsen, 1933).

The climate at Boulder City was characterized by a daily cycle of temperature and humidity, the maximum temperature, found at about 2 o'clock each afternoon, being markedly uniform from day to day. It was at this time that most of the tests were carried out; the mean air temperature during them was 35.3°C. (96°F.), with a maximum at 43°C. In the shade the effective temperatures (Houghten and Yagloglou, 1924) were 27°C. in still air and 26°C. in wind, never exceeding 29°C. The mean relative humidity was 12 per cent, and was above 30 per cent only on one day. The wind velocity was high and fairly steady.

The general plan was to measure, under various conditions of exposure, the changes of heat content of the body (by thermometers), the rates of heat production (by consumption of oxygen), the rates of heat gain from radiation, and the rates of heat loss through evaporation. For present purposes, the heat content of the body might be said to represent the resultant of heat production + radiation received − heat lost in evaporation. In this equation gains and losses by conduction and convection are omitted. In many instances the atmospheric temperature was very near the mean

[1] The author is indebted to the other members of the party for their collaboration: D. B. Dill, F. G. Hall, H. T. Edwards, R. A. McFarland, F. Consolazio, and D. B. Dill, Jr. Laboratory space, and certain meteorological and hydrographic data, were obtained through the kindness of Mr. John Page, Commissioner, Mr. Ralph Lowry, Resident Engineer, Mr. Frank Dunbar, Hydrographer, and other members of the U. S. Bureau of Reclamation.

skin temperature; when this occurred, the equation as presented will be shown to balance with fair accuracy.

SUBJECTS AT REST. *Body temperatures.* Rectal temperatures were measured by ordinary clinical thermometers. It has been shown by Burton (1935) that under some conditions the heat content of two-thirds of the mass of the body is proportional to the rectal temperature. The heat content of the other one-third may be considered as an algebraic function of mean surface temperature. Surface temperatures were

TABLE 1

Body temperatures (T) in °C. measured in each subject under various conditions

Each surface temperature was the average of 9 or 10 areas, and the number of averages obtained is indicated in parentheses.

	A	C	D	H	M	MEAN
Mean surface T in shade	34.4 (9)	34.1 (12)	34.3 (5)	34.2 (13)	32.4 (5)	33.9
Rectal−surface T in shade	2.6 (9)	3.1 (12)	2.8 (5)	3.4 (13)	4.5 (5)	3.4
Mean rise of rectal T sitting in sun	0.5 (2)	0.1 (3)	0.0 (2)	0.1 (3)	0.1 (2)	0.2
Rectal−surface T sitting in sun	2.9 (6)	1.8 (9)	3.2 (4)	2.7 (5)	2.3 (5)	2.5
Mean rise of rectal T in one-hour walk	1.2 (19)	0.75 (29)	0.7 (14)	0.6 (4)	0.8 (6)	0.7
Rectal−surface T in first 25 min. after exercise	4.6 (9)			4.8 (4)		4.7
Rectal−surface T in second 25 min. after exercise	3.8 (8)			4.1 (3)		3.9

measured by thermocouples held (spread by tension wires, Aldrich, 1928) against the skin and allowed to rest for 5 to 10 seconds before each reading was made on the galvanometer. The couple and the "cold" junction (in a vacuum flask) were moved about (outdoors), while a fixed galvanometer was read (indoors) some 12 m. away, at each signal on a buzzer. No great significance attaches to the absolute temperatures reported; for the most part only the magnitudes of the changes in surface temperature were desired.

It was found that the surface temperatures varied little with time in subjects at rest and in the shade. Even in surface areas exposed to direct radiation, temperatures usually rose less than 2°C., and exceeded the rectal

TABLE 2

Rates of heat exchanges, and trial balances, in Calories per hour

Production (6) was computed from oxygen consumption rates, solar radiation (7) from table 3, other gains (8) by difference of "Sum" − "Gains" (5-6-7).

(1)	(2) SUBJECT	(3) LOSS BY EVAPORATION	(4) ACCUMULATION	(5) SUM	(6) GAIN BY PRODUCTION	(7) GAIN BY RADIATION	(8) OTHER GAINS
Sit in shade at 37.3°C.	C	220	3				
	D	230	0				
	H	255	0				
	M	290	1				
	Mean...	249	1	250	90	0	160
Sit in sun at 37.4°C.	A	380	37				
	C	383	7			186	
	D	450	2			133	
	E	420	0				
	H	340	7			84	
	M	440	8			168	
	Mean...	402	10	412	90	143	179
Walk in shade at 37.0°C.	A	516	49				
	C	545	52				
	E	485	17		420		
	M	488	50		467		
	Mean...	508	43	551	444	0	107
Walk in sun at 35.3°C.	A	540	75		474	128	
	C	640	48		554	197	
	D	595	48		560		
	E	500	19		447	70	
	H	530	37		540		
	J	710	62				
	M	700	65		422	232	
	Mean...	603	51	654	500	157	−3

temperature only on nearly horizontal surfaces, so long as the thermocouple was shaded during the measurement. This coolness of the skin in outdoor conditions is intelligible in connection with the large rates of evaporation that prevailed on these surfaces. Blood might carry heat away from the

radiated areas to the non-radiated surfaces for dissipation, as has also been found under laboratory conditions (Pinson). The mean surface temperature (table 1) in the sun exceeded that in the shade by less than 1°C.

The upshot is that one can disregard surface temperatures in estimating to an accuracy of 15 per cent the amount of heat accumulated in the body during a period of exposure to desert conditions; and in the measurements reported, heat accumulation has been estimated from the rectal temperature alone, using the coefficient 0.83 for the specific heat of the weighed individual.

Heat production was not measured in the desert on our subjects at rest. It may be presumed from the measurements of others (*e.g.*, Houghten et al., 1931) carried out under a variety of atmospheric temperatures, that the subjects who were sitting with very little muscular activity produced between 80 and 100 Calories per hour (table 2). It was the smaller element in gain of heat in all the tests performed, since radiation from the sun, and sometimes also convection and conduction inward from the air, exceeded it in magnitude.

Evaporation rates were measured by weighing the individuals at 20 or 30-minute intervals on a balance (silk scales) sensitive to 10 grams. The subjects in most of the tests wore shorts, socks, and shoes, an amount of clothing which remained dry and was insufficient to complicate the evaporation processes. In the dry atmosphere, no moisture was visible upon exposed areas, all the moisture lost from the body being evaporated. No corrections have been made for the diminution of weight represented by carbon exhaled, since this constituted only 5 to 10 per cent of the loss at rest, and much less of the loss (3 per cent) when exercise was taken.

Solar radiation to the body was estimated by two means; the first method was to measure simultaneously the rates of evaporation in four subjects, two of whom were sitting in the sun, the others in the shade of a canvas awning. Since the subjects were almost side by side, little or no differences in convection, in conduction, and in radiation from the ground supervened. In several of the tests evaporation proceeded at double the rate in the sun that it did in the shade. In 6 paired tests (table 3), each pair representing the same subject on different days, the mean difference in evaporation during exposure to the direct sun amounted to 242 grams (140 Calories) per hour. It is an approximate measure of the amount of radiation received from sun and sky by the average subject sitting in the sun.

The second method was to find with a radiometer how much radiation was reflected from various skin areas. The radiometer, which was somewhat like the one used by Martin (1930), was a blackened unshielded thermopile enclosed in a portable vacuum bottle. In the silver-plated thermopile, the cold junctions were only 4 mm. distant from the hot junctions, so that equilibrium was obtained within 5 seconds of exposure

of the thermopile to radiation. Paired readings were made; the first with the vacuum bottle covered by a box that shut out radiation; the second with the box removed and the thermopile pointed toward the skin of the subject and 21 cm. from it. A variety of angles of reflection was measured, but in no case were sufficient measurements taken to allow integration of the amounts of reflection for the whole body or for considerable areas of it. No absolute calibration was made. The measurements compared the absorptions of radiation upon a reflector of etched aluminum foil, clothed areas, tanned areas, and areas exposed at various incident angles.

Radiation reaches the earth at the rate of 1.4 small calories per cm.^{2} per minute (Abbot, 1911). The shadow areas of the seated subjects amounted to 2000 to 3000 cm.^{2}, and hence the maximum radiation impinging was 250 Calories per hour. From unclothed areas roughly half as

TABLE 3

Rates of evaporation (loss of body weight) in grams per hour

For each subject single measurements are listed separately

SITTING	C	C	D	H	H	M	MEAN INCREASE
In shade	450	330	400	450	430	530	
In sun	730	690	630	570	600	820	242
Clothed in sun		560			420		110
WALKING	**A**	**A**	**C**	**E**	**E**	**M**	**MEAN INCREASE**
In shade	870	910	940	860	810	840	
In sun	1,010	1,210	1,280	960	950	1,240	237
Clothed in sun			1,100				160

much radiation was reflected at all angles as from comparison reflectors; therefore about 125 Calories per hour were absorbed. This value resembles 140 Calories derived by the first method.

In conjunction with both methods of radiation measurements it may be believed (Abbot, 1911) that the intensity of radiation from sun and sky was constant within 10 per cent. The variation is least in climates where low humidities prevail. In a few tests thin clouds appeared between the sun and the subject. It is well known, however, that clouds do not shut off a large proportion of radiation, and markedly faster evaporation rates were found in subjects exposed to clouded radiation as compared to those shaded by awning or building.

In a few tests, subjects were clothed fully in light cotton materials, and exposed to full solar radiation (table 3); considerable protection against gain of heat from radiation ensued. Radiometric studies likewise showed

that the amount of reflection from the surface might be doubled when the skin was covered by white cotton, as Martin (1930) also noted.

Before they could endure these daily prolonged exposures to intense solar radiation, the subjects were, of course, deeply tanned. As is well known, tan is no protection against absorption of heat, and less radiation was found to be reflected under these conditions. Visible moisture never appeared on the skin to complicate the radiometric measurements.

Radiation was a sufficient factor to increase by 65 per cent the total amount of evaporation from subjects at rest (table 2). Behavior that protected even a small area of skin against direct solar radiation was a distinct relief.

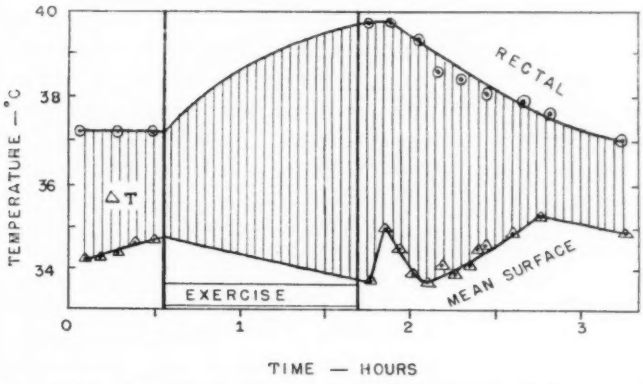

Fig. 1. Surface and rectal temperatures, taken indoors, before and after 77 minutes of tennis (July 4, subject A). The mean surface temperature is the average of thermocouple measurements at ten standard points on the skin. Outdoors: 39.4°C. dry bulb, 10 per cent humidity; indoors; 32.7°C., 30 per cent. Sweat accumulated on the skin in the first few minutes after exercise.

Trial *balances of heat* (table 2) may be constructed from the partial measurements at hand. Heat losses were measured only as evaporation (column 3). The amount of heat gained by the body was at least equal to that lost by evaporation plus that stored (accumulated, 4). The heat produced (6) was computed from oxygen consumptions and CO_2 productions. The heat gained by radiation from sun and sky (7) was obtained by the above method. Heat gained (or lost) from the surroundings (conduction, convection, and ground radiation) could be obtained (by a method mentioned below) from the variation of evaporation rates with atmospheric temperatures; it has not been inserted in the table. To these rates of loss (3) have been added (5) the rates of heat accumulation (4). These partitions of heat exchanges assign mean values to the rates, under

the particular temperatures and other conditions of the atmosphere and of activity prevailing.

During exercise. Exercise periods were at the hottest part of the day, and ordinarily consisted of walking at a rate of 6 to 7 km. per hour, for one or two hours, during which the subject came into the laboratory at 20-minute intervals for measurements. In other cases the subjects played tennis at vigorous rates for about one hour.

Body temperatures. Changes of surface temperature (fig. 1, table 1) were such as to be almost negligible in total heat balance. While some areas such as the legs became cooler during exercise and exposure, other

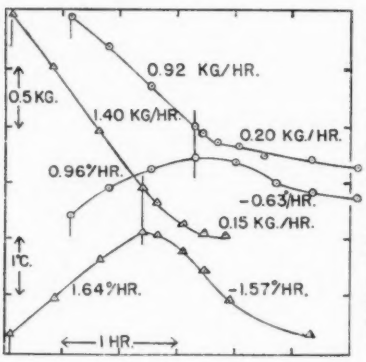

Fig. 2

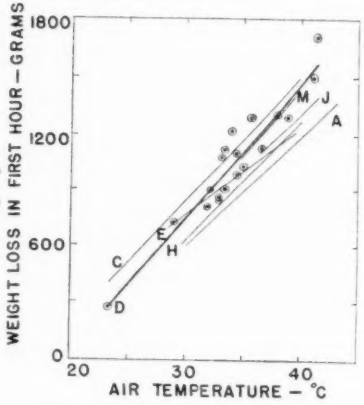

Fig. 3

Fig. 2. Body weights and rectal temperatures of subject A during (outdoors) and after (indoors) two exercise periods. Outdoors: June 17th ⊙, 32.6°C., 7 per cent humidity; July 2nd △, 41.4°C., 7 per cent. Indoors: June 17th, 29.0°C., 57 per cent; July 2nd 32.2°C., 52 per cent.

Fig. 3. Correlation of weight losses and atmospheric temperatures during daily walk at 7 km. per hour in subject D. Similar correlation lines are indicated for each of the other six subjects.

areas on the trunk became correspondingly warmer. The mean surface temperatures appeared to decrease (by less than 1°C.) during exercise, as Benedict and Parmenter (1929) also reported.

Meanwhile rectal temperatures rose considerably on very hot days and especially in some subjects. Thus the gradients or differences of temperatures (table 1) between deeper portions of the body and the surface, became greater, and represented an economy in the transport of heat by the blood.

The heat accumulations may be again taken to be proportional to the increases in rectal temperature; they amounted (fig. 2) to as much as 130 Calories in the first hour of exercise. In other subjects, however, extremely

small accumulations of heat resulted (table 2), even though equally large amounts of heat were being produced and lost.

Heat production was ascertained in each of the subjects at his standard rate of walking (table 2). The greatest rate of heat production maintained for a one-hour period was that measured in subject M during trotting on a hot day; 700 Calories per hour.

Evaporation rates that were measured may be averaged for each subject (table 2) since he walked at the same rate in all tests included (see also fig. 3).

The maximum rate of evaporation was not reached immediately in some subjects, so that in the first 20 minutes of exercise the rate of weight loss was 10 to 20 per cent less than in the two succeeding 20-minute periods. Since the weight loss represents (to the extent of 80 to 90 per cent in most cases) production of sweat, it may be said that there was some lag in the initiation of sweating. Heat production (oxygen consumption) also is well known to increase in exercise with a similar lag. Some sweating was usually going on even before exercise began; and in a great many tests on some subjects, and in all tests on two of them, no lags occurred.

The rates of weight loss during exercise have already been discussed in relation to water balance (Adolph and Dill, 1938). The maximum rates of evaporation observed in each of the seven subjects were from 1290 to 1700 grams per hour. These correspond to heat losses of 750 to 990 Calories per hour.

The effect of *solar radiation* was measured during exercise by comparing the rates of weight loss in paired tests (table 3); the mean difference was 237 grams or 137 Calories per hour in the heat dissipated by evaporation. The tests were as strictly comparable as could be carried through; one of the two subjects walked for an hour in the shade of a long dormitory building while the other subject walked in a parallel path 4 or 5 meters distant in the sun. Later each subject walked at the same rate, but on the opposite track; hence these paired tests were for the same subject on the same day.

One might conclude that the amount of exposure to radiation from sun and sky was approximately the same in the walking subject and in the sitting subject (table 3). While this amount might also vary somewhat with zenith distance of the sun, actually all the tests were sufficiently near the same time of day so that changes of declination were apparently negligible. The individuals exposed 2500 to 4000 sq. cm. to the sun, as measured in shadow areas while standing (noon to 3 p.m.).

Again the effect of thin clothing was noted on one subject (table 3); less evaporation was required to preserve the heat balance. One may conclude that cotton clothing is a considerable asset in continued exposures to direct sunshine.

Respiratory heat losses meant that the air breathed was considerably cooled instead of being warmed, as in most climates. Measurements of expired air temperatures all fell between 33 and 34°C. When the temperature of the inspired air was 43°C., about 6 Calories per hour were required to cool the 2200 liters of air breathed in one hour, an evaporation of water of about 10 grams. Much more water than this was actually evaporated from the respiratory passages during the hour of exercise, for at this ventilation rate 75 grams could be taken up by the air. In other words, the blood is cooled at the surfaces of the air passages, even though the air is cooled at the same time. Determinations of alveolar carbon dioxide ten-

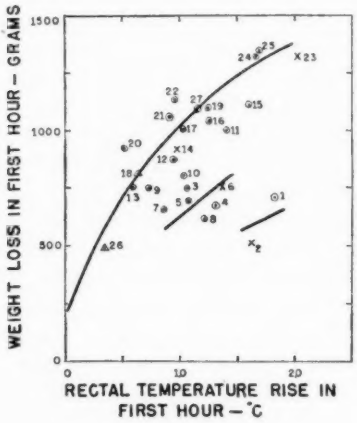

Fig. 4

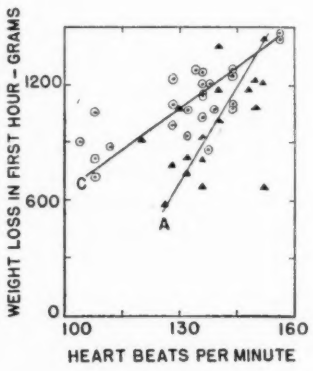

Fig. 5

Fig. 4. Correlation of weight losses and rectal temperature gains in first hour of daily exercise in subject A. The days are numbered serially. Walk ⊙, tennis ×, walk in shade, △.

Fig. 5. Correlation of weight losses and maximum pulse rates during daily walk at 7 km. per hour in two subjects.

sions were made; these yielded no evidence of over-breathing, either at rest or in exercise. Hence, there was nothing to be designated as panting, no attempt to use ventilation as a means of increasing evaporation.

In exercise tests (table 2) two heat exchanges were always measured together, namely, rate of evaporation and rate of *rise of body temperature*. Correlations of the two quantities (fig. 4) indicate that in the acclimatized individual, great accumulations of heat in the body occurred only with high rates of evaporation. Or, sweating did not dispose of so large a fraction of the heat when the rates of heat exchange were high. The rate of sweating approached an asymptote, leaving a larger excess of heat in the body.

Subject A (fig. 4) showed a marked and progressive increase in the rate of sweating for a given rise of rectal temperature. After ten days this acclimatization no longer increased much.

Those subjects that suffered only small rises of rectal temperature had large ratios (table 4) of heat lost by evaporation to heat accumulated in the body. The greatest accumulations were in subject A who perspired with difficulty and suffered discomfort. The smallest were in subject E who perspired readily, though he also walked at a slower pace.

A preliminary test was carried out during the previous winter upon each subject, walking on a treadmill under somewhat comparable conditions (44°C., 9 per cent humidity, fan) in Boston. In spite of the fact that the rates of sweating were less than in the desert, the amounts of heat accumulated were great as compared with those in the desert. This may be

TABLE 4

Ratios of heat lost by evaporation to heat accumulated in the body during walking

	A	C	D	E	H	J	M	MEAN
Number of tests* in Boulder City	20	27	12	18	3	3	6	13
Minimum ratio	3.7	8.7	8.2	8.8	10.6	7.7	7.0	7.8
Maximum ratio	11	45	26	85	18	14	17	31
Mean ratio	7.7	15.3	13.5	38.6	14.9	11.5	11.5	16.1
Mean evap. rate,* gm./hr	930	1,100	1,025	860	910	1,220	1,210	1,035
Ratio in Boston test	2.7	17.8	6.3	6.5	6.7		9.6	8.3
Boston evap. rate, gm./hr	640	820	770	810	590		990	770

* The same tests are represented in the last quarter of column 3 in table 2.

taken as evidence of less readiness (or of disability) to sweat in mid-winter. The same individuals were then even more poorly acclimatized than upon the first day of exposure in the desert.

Atmospheric temperatures were a factor in the rates of evaporation in uniform conditions of exercise (fig. 3), as might be anticipated. The rates were such that for each degree C. rise in air temperatures, 62 grams (36 Calories) more were evaporated in an hour. Similar correlations were found for each of the subjects. Diverse atmospheric conditions give slightly different slopes to the correlation line (Adolph, 1923; Houghten et al., 1931; Winslow et al., 1937). But for any one condition the line tells how much extra water is required for sweat formation, according to the temperature encountered. The increased evaporation represents the increment of cooling (and hence of heat gain) due to exchanges by *convection and conduction*. The correlation holds both below and above skin

temperatures, in the one case convection being away from the body, and in the other case toward the body. This may be compared with the data of Herrington et al. (1937) under indoor conditions of radiation and convection.

The balance between measured rates of heat gain and measured rates of heat loss turns out to be surprisingly close in the exercise experiments (table 2). The data are most numerous for the tests in the sun, and it so happens that the mean air temperature for the 27 days involved in them was very close to the probable mean surface temperature. Hence other net gains and losses of heat (by conduction and convection, column 8) were probably cancelled.

After exercise. The experiments furnished opportunity to study the rates of cooling of the body. Skin temperatures did not vary much after exercise (fig. 1) so that again rectal temperatures could be used to calculate the amounts of heat present at various times. All observations during cooling (recovery) were made indoors under different conditions than those in which the exercise had been taken; and in no instance, therefore, was heat being gained by radiation, conduction and convection. The rates of cooling were such that the least time required to lose the amount of heat that had been gained during the experiment was one hour. In general, heat was lost faster if the accumulated heat was greater, so that, within the accuracy of the data obtained, all body temperatures would be restored to the original in 1.0 to 1.5 hours.

During periods of recovery changes of body weight were also measured. These data, however, cannot be taken to represent evaporation only, since the subjects washed and rubbed down with towels, and it was found that considerable quantities of excreted moisture were removed in that way.

The circulation. Pulse rates were taken in all exposures; very marked increases prevailed in exercise. Subject C, sitting in the sun had a pulse rate of 84 beats per minute, walking in the shade, 120, and walking in the sun, 144, on days of equal temperature. Marked correlations resulted between pulse rates and rates of sweating (fig. 5) in all the subjects.

Arterial pressures were measured in some instances during walking. In one subject before acclimatization the systolic pressure regularly increased to 200 mm. of mercury, the diastolic pressure decreased below the resting value.

In cool climates no such increases of pulse rate and pulse pressure are found in walking. Hence, it may be said that the circulation was speeded, in the absence of cooling by any process other than evaporation. It may be estimated that if the blood cools 4°C. at each trip to the body surface, then about 2.5 liters of blood circulated to the surface per minute in order to transport the 500 Calories per hour that were produced. In addition

to this there may be some transfer of heat from radiated surfaces to non-radiated surfaces.

Two of the subjects noted a tendency toward development of œdema in the legs during walks at high temperatures. Whether the development of edema was more rapid in the ankles and legs on hot days as compared with cool ones, was not certain, but such was the impression.

COMMENT. A rough estimate of the total amounts of heat gained and lost by the body would be 3000 Calories per day. More than half this much was lost by evaporation alone, since 2600 cc. more water (= 1500 Calories) was metabolized per day in the desert than in winter (Adolph and Dill, 1938). The daily difference of rectal temperatures (taken upon rising each morning) was ± 0.13°C., representing 8 Calories net retained or lost. From this it may be concluded that of the total amounts of heat handled by the human body in 24 hours, the gain differed from the loss by only ± 0.3 per cent. On this basis, heat balance may be said to be regulated about ten times as accurately as water balance in 24-hour periods (Adolph and Dill, 1938). The contrast is as great over shorter periods, for water (which in the desert departed from balance entirely on the side of deficits) stayed unbalanced for hours; while heat (which appeared only in excesses) was continually lost, and recovered balance with considerable precision in one hour after cessation of exercise.

Several evidences of changes in the subjects during the successive days of exposure to high temperatures were revealed. It should not be concluded that these were responses to high temperatures, since the element of training to the exercise performed was also present. One was in the ability to produce sweat (subject A). Three other subjects showed increases in the proportion of the heat lost during exercise in summer over that found in winter (table 4).

Adjustments occurred in the circulation after a series of exposures to high temperatures during exercise. This is indicated only in determinations of pulse rate and arterial pressure, and in the intensity of pulse-throbbing in the ears, so far as present observations go. Differences in volume output of the heart and in the distribution of the blood pumped by the heart may be concerned.

Evidences of individual differences were chiefly of two sorts: first, differences in ratio of heat lost by evaporation to heat accumulated in the body (table 4), and second, differences of evaporation rate relative to pulse rate (fig. 5). Evaporation rates were not limited by the evaporating power of the air in any of the conditions studied, since the skin stayed dry; in other words, the air was capable of taking up moisture still faster than the moisture was presented to it. One may think of the sweat glands and their control as limiting the amount of moisture that is presented for evaporation. The ability of diverse individuals to withstand desert conditions appeared

to vary largely with the rates of sweat production, for with increased sweat production there was less heat accumulated in the body. Subjects who did not accumulate much heat were more comfortable, felt more fit, and were able to continue exercise for longer periods. Even these individuals, however, were limited in the rates with which they could do work for any considerable time.

One limitation to work may be the rate of sweating. Evaporation rates rarely involved heat losses of more than 800 Calories per hour. In the sun 140 Calories were received by radiation, leaving only 660 Calories available to dissipate the heat produced in exercise. While still more evaporation might conceivably occur and so keep the body cool at still higher rates of heat production, actually, consistently higher rates of evaporation have not been found by anyone.

Individuals in the desert seek shade, refrain from violent exercise, wear protective clothing, and choose the cooler conditions whenever possible; all are factors in preserving heat balance. It is well known that travelers through the desert habitually walk at night when possible (Chase, 1919).

The changes with acclimatization, measured in rates of evaporation and in rates of heat accumulation in the body, are significant factors in enduring a given hot atmosphere. Shaklee (1917) ascertained that monkeys were killed within an hour of exposure to direct solar radiation, while others that were given shorter repeated radiations until rapid evaporation developed, later survived indefinite exposure. It has been reported (Yaglou and Drinker, 1928) that considerably higher air temperatures are preferred by human beings in summer than in winter. With this preference may be related the decreased basal heat production (Lockwood and Griffith, 1938), and the increased ability to sweat in summer as compared with winter.

SUMMARY

Partial heat balances were measured in desert conditions by comparing rates of heat production, of gain by radiation from the sun, of loss by evaporation, and of heat accumulation.

In rest radiation may add heat to the body faster than heat is produced chemically. In exercise, along with high rates of heat production, radiation and convection added heat, so that the loss by evaporation alone exceeded heat production.

Recovery from exercise required 1.0 to 1.5 hour for the dissipation of the heat that had accumulated.

During exercise the pulse rate and the systolic arterial pressure increased with rates of heat loss, indicating that the circulation took a prominent part in the increased rates of heat exchange.

Changes with successive days of exposure occurred in the rates with

which evaporation took place, in heat accumulated, in pulse rates, and in choices of behaviors that prevented the access of heat to the body.

Individual differences were noted which suggest that not all persons are equally able to preserve heat balances.

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CHANGES WITH AGE IN THE RENAL FUNCTION IN ADULT MEN

I. Clearance of urea. II. Amount of urea nitrogen in the blood. III. Concentrating ability of the kidneys

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Three functions of the kidneys have been known to exhibit variations in normal individuals: I, the ability of the kidneys to remove urea from the blood; II, the amount of urea or urea nitrogen present in the blood; III, the ability of the kidneys to concentrate urine, as exhibited by the specific gravity.

Variations in the clearance of urea may depend on the volume of urine (1, 2), on the size of the body (1, 3), on posture and exercise (4), on unusual diet (5), on drugs (6), and on the presence of disease (7, 8, 9). The amount of urea nitrogen in the blood differs in young normal people (10) and in people with renal disease (7, 8, 11, 12, 13). Variations in the concentrating ability of the kidneys occur likewise (14, 15, 16, 17).

To these factors should be added the influence of age, unattended by any concomitant disease. Observations on the three functions mentioned have been made primarily in young normal individuals. There have been no systematic descriptions, decade by decade, of their course over the period of mature life.

The study now to be reported undertakes the analysis of these functions in normal men over 40 years of age. Its purpose has been twofold: 1, to establish average values and to ascertain the range of variations from these averages, and 2, to measure the rate of change at different ages, of these functions. The observations have been made on the same group of individuals.

Subjects. The subjects were normal men, twenty in each decade from 40 to 89 years, two of 91 years, and one of 101 years. Measurements of certain of their functions have been described in previous reports (18).

In view of the possibility of obstruction to urinary flow through the urinary passage and consequent retention of urine in the bladder, a factor bearing on the urinary volume obtained and the quantity of urinary urea excreted, the size of the prostate gland was estimated by digital examina-

tion. In the observations on the clearance of urea in four subjects, the fractional urinary volumes and the presence of prostatic enlargement indicated that this factor deserved consideration (table 1, nos. XXXII, LVI, LXXVI, XCVIII). The clearance of urea and the amount of urea nitrogen in the blood were measured in all subjects. The concentrating ability was measured in thirty-eight men; their ages ranged from 40 to 89 years, of whom eight were in the 5th decade, four in the 6th, eight in the 7th, seven in the 8th, and eleven in the 9th.

METHODS. The men were admitted to the hospital in the early afternoon of one day and discharged on the afternoon of the second day.

Clearance of urea. The procedure of the urea clearance test was carried out on the afternoon of the day of admission. In a few subjects, it was performed on the morning of the second day. The subjects were usually at rest during the procedure. The samples of blood were taken about three hours after the noonday meal. The content of urea nitrogen in the samples of blood and urine was measured by the gasometric urease method (19).

Meals, containing the ordinary amount of protein, time of day, and posture, do not affect this function significantly (4). Other factors that have been shown to be of some importance are unusual amount of protein in the diet (5) and the formation of ammonia by the kidney (8, 20). The latter factor, being of large magnitude only in those subjects with severe acidosis or severe cystitis or in users of low protein diet, may be ignored in normal individuals.

The analytical technique for measuring the ability of the kidneys to clear urea from the blood has been described (2, 3); the test measures the amount of blood cleared of urea per minute by the kidneys. The results are given in per cent of mean normal clearance exhibited by young normal men. The mean normal value for young adult individuals is 54 ccm. of blood cleared per minute (standard clearance) when the volume of urine is low (under 2.0 ccm. per minute); and 75 ccm. cleared per minute (maximum clearance) when the volume of urine is high (above 2.0 ccm. per minute). If these mean normal values are taken to be 100 per cent, comparative tests may be given in percentages. The range of variation in young normal individuals has been observed to be 75 to 120 per cent of the mean normal values (2). A correction is made for body size when the subject is much above or below average adult stature (3).

Urea nitrogen of the blood. Blood for chemical analysis was drawn from an arm vein; it was usually obtained in the afternoon. The time of day at which the blood is taken, in normal individuals on the usual diet, does not affect the level significantly (10). The amount of urea was measured by the gasometric urease method (19).

Concentrating ability of the kidneys. The concentration test of Addis

and Shevky (14) was utilized. The subject was placed on a dry diet for 24 hours. No fluid was taken from the morning of one day to the morning of the next. During the second 12 hours of the dry period, the urine was collected and the specific gravity of the specimen measured. No special preliminary diet was given. Correction of the specific gravity was made for the quantity of protein present in the urine (observed specific gravity 0.003 × protein in per cent), according to the observations of Lashmet and Newburgh (15, 16). The urinometer and modified Westphal balance provide accurate readings of 0.0005 (17). The urinary protein was measured by the method of Shevky and Stafford (21), modified by MacKay (22).

RESULTS. I. *Clearance of urea by the kidneys.* The results are presented in several ways:[1]

a. The mean values of successive five-year groups indicate that a decline in this function takes place with age. In men of 40 to 44 years, the mean value was 96.1 per cent of the average normal clearance. The means in later five-year classes declined irregularly to 54.6 per cent at 85 to 89 years. The decline was more orderly if the results were collected in ten-year groups. The decline from 65 to 89 years was approximately four times that from 40 to 64 (table 1, fig. 1 A).

The individual values ranged from 127 per cent in a man of 55 years to 19 per cent in a man of 85 years (table 1). In three men, values above 120 per cent were found; they were 40 to 55 years of age. In fourteen men, values below 50 per cent were found; they were 65 to 101 years of age. The scatter was smaller in the younger groups. In this series, it resembled that found in young normal adults (2), (fig. 1 A).

The standard deviation of individuals from the mean of their age groups was larger in the elderly (fig. 1 B) because some older men showed a decline in clearance, while others showed little or none. The standard deviation was 10.8 per cent of normal, or 12 per cent of the mean in the 45–49 year class; it was 32.9 per cent of normal, or 60 per cent of the mean in the 85–89 year class. Grouped in decades, the standard deviations were smaller (fig. 1 B). For the whole series of one hundred men, the mean was 78.5; the standard deviation was 23 or 30 per cent of the mean.

b. The mode and the median decreased with age. This fact, apparent in figure 1 A, is shown more clearly if the data of table 1 are arranged in a histogram.

c. The linear equation for the relationship of urea clearance and age is:

$$\overline{UC} = 136.6 - 0.912 \times A$$

where $\overline{UC}$ = urea clearance in per cent of normal clearance values and A = age in years.

[1] References to the statistical methods employed are given in a previous report (18).

TABLE 1

Certain functions of the kidneys

Data for calculation in normal men over 40 years of age

AGE GROUP	SUBJECT NUMBER	AGE	UREA CLEARANCE IN PER CENT OF AVERAGE CLEARANCE IN YOUNG NORMAL MEN (2)			UREA NITROGEN IN 100 CC. BLOOD	CONCENTRATION TEST*			REMARKS†
							12 hour urine			
			1st hour	2nd hour	Average hourly		Specific gravity (non-protein)	Volume	Protein	
years		*years*				*mgm.*		*cc.*	*gm.*	
40–44	I	40	110	89	99	15.9				
	II	40	141	107	124	10.5	1.0265	344	0	
	III	41	104	119	111	11.0				C+
	IV	41	116	103	109	11.1				
	V	41	69	74	72	15.2	1.0347	256	0	
	VI	43	89	85	87	11.7				P+
	VII	44	68	81	75	16.0	1.0258	303	0	
	VIII	44	87	77	82	11.6				
	IX	44	125	94	110	13.6	1.0293	510	0	
	X	44	105	79	92	12.7				
45–49	XI	45	103	94	98	11.3	1.0362	491	0	
	XII	45	103	99	101	12.7				
	XIII	45	112	48	80	12.0	1.0280	445	0	
	XIV	46	95	84	90	8.1				
	XV	46	118	94	106	15.8				
	XVI	47	83	65	74	13.9				
	XVII	47	114	105	110	14.2	1.0282	425	0	
	XVIII	48	112	90	101	7.0				
	XIX	48	95	75	85	13.6				
	XX	49	98	89	94	16.5	1.0260	423	0	
50–54	XXI	50	78	94	86	13.6				
	XXII	51	76	71	74	14.3				
	XXIII	51	60	47	54	13.7	1.0263	437	0	
	XXIV	51	137	104	121	15.3				
	XXV	51	56	55	55	18.1				P+
	XXVI	53	86	94	90	13.9				A+, C+
	XXVII	53	94	95	95	11.7				
	XXVIII	53	63	70	67	16.7				
	XXIX	53	97	98	97	15.4				
	XXX	54	64	72	68	14.7				
55–59	XXXI	55	104	117	111	12.9				A+, C+
	XXXII	55	172	81	127	10.2				
	XXXIII	55	90	107	99	14.6	1.0316	248	0	
	XXXIV	56	71	67	69	14.9	1.0258	436	0	
	XXXV	57	80	52	66	13.2				C+
	XXXVI	58	111	86	99	14.1				P++
	XXXVII	58	93	78	85	14.6				

*Concentration test of Addis and Shevky (14); also cf. Alving and Van Slyke (17), Shevky and Stafford (21).

† A albumin in urine: + faint trace, ++ trace, +++ heavy trace.

C cellular elements in urine: + rare, ++ few, +++ frequent.

P hypertrophy of prostate: + mild, ++ moderate, +++marked.

TABLE 1—*Continued*

AGE GROUP	SUBJECT NUMBER	AGE	UREA CLEARANCE IN PERCENT OF AVERAGE CLEARANCE IN YOUNG NORMAL MEN (2)			UREA NITROGEN IN 100 CC. BLOOD	CONCENTRATION TEST* 12 hour urine			REMARKS†
			1st hour	2nd hour	Average hourly		Specific gravity (non-protein)	Volume	Protein	
years		*years*				*mgm.*		*cc.*	*gm.*	
55–59	XXXVIII	59	84	126	105	17.2				
	XXXIX	59	62	80	71	12.0	1.0310	298	0	P++
	XL	59	79	85	82	14.4				C+
60–64	XLI	60	97	102	99	11.9				C+
	XLII	60	114	108	111	15.2	1.0308	312	0	
	XLIII	61	102	79	90	8.3	1.0253	328	0	
	XLIV	61	60	88	74	9.2				
	XLV	61	146	80	113	14.5				P++
	XLVI	61	79	83	81	18.3				P+
	XLVII	63	95	67	81	17.5	1.0284	320	0	P++
	XLVIII	63	68	68	68	15.1				
	XLIX	64	102	107	105	13.4				
	L	64	79	67	73	17.6	1.0296	210		P+
	LI	64	77	73	75	11.0				
	LII	64	101	92	96	11.8				
	LIII	64	68	65	67	12.9	1.0280	230	0	A+, C+, P++
65–69	LIV	65	47	40	44	19.5				
	LV	65	60	60	60	14.2				A+, C+
	LVI	66	99	86	93	20.2	1.0278	420	0	A+
	LVII	66	96	83	90	11.3				
	LVIII	67	85	77	81	13.8				C+, P+
	LIX	68	74	76	75	12.3	1.0230	239	0	P+
	LX	69	87	55	71	14.5	1.0284	426	0	P+
70–74	LXI	70	74	69	71	9.5				
	LXII	70	65	71	68	13.1				A+, C+
	LXIII	70	55	63	59	21.2				
	LXIV	71	35	39	37	16.2				A+, C+, P++
	LXV	71	86	71	78	17.9				
	LXVI	71	55	41	48	11.3				P++
	LXVII	71	81	76	79	14.3	1.0252	342	0	C+, P+
	LXVIII	72	55	85	70	17.7	1.0236	521	0	C+
	LXIX	72	91	51	71	12.6	1.0327	230	0.066	
	LXX	72	47	51	49	14.7	1.0322	288	0	P+
	LXXI	72	68.2	68.2	68.2	14.5	1.0183	465	0	A+, P+
	LXXII	73	112	127	119	11.5				A+, P+
	LXXIII	74	72	61	67	15.5				A+, C++, P+
75–79	LXXIV	75	62	75	68	16.2				A+, C+, P++
	LXXV	75	76	56	66	12.8				P+
	LXXVI	76	23	55	39	16.5	1.0179	360	0.058	A+, C+, P+

TABLE 1—*Concluded*

AGE GROUP	SUBJECT NUMBER	AGE	UREA CLEARANCE IN PER CENT OF AVERAGE CLEARANCE IN YOUNG NORMAL MEN (2)			UREA NITROGEN IN 100 CC. BLOOD	CONCENTRATION TEST*			REMARKS†
							12 hour urine			
			1st hour	2nd hour	Average hourly		Specific gravity (non-protein)	Volume	Protein	
years		*years*				*mgm.*		*cc.*	*gm.*	
75–79	LXXVII	77	80	64	72	15.3	1.0272	234	0	
	LXXVIII	77	52	93	72	13.4				P++
	LXXIX	77	28	75	51	14.9				C+, P+
	LXXX	78	49	63	56	15.4				A+, C++
80–84	LXXXI	80	55	50	52	12.5	1.0241	379	0	A+, C+
	LXXXII	80	58	50	54	20.5	1.0230	326	0	C+
	LXXXIII	80	61	123	92	12.2	1.0252	242	0	P++
	LXXXIV	80	78	96	87	19.5	1.0221	393	0	A+, C+, P+
	LXXXV	81	107	92	99	12.4				P+
	LXXXVI	81	45	47	46	21.5				A+, C+, P+++
	LXXXVII	82	31	58	44	15.0	1.0224	405	0.087	A+++, C++, P+++
	LXXXVIII	82	109	50	80	11.0				A+++,C+++, P+
	LXXXIX	82	38	86	62	17.5				A+, C+++, P+
	XC	82	24	49	36	22.6				A+++,C+++, P+
	XCI	82	41	66	54	14.4	1.0270	283	0	A+++,C+++, P+
	XCII	83	59	56	57	21.2	1.0240	438	0	A+, C+, P++
	XCIII	84	59	40	50	14.7				P+
	XCIV	84	88	66	77	13.7				P+
85–89	XCV	85	109	113	111	9.3	1.0210	225	0	A+++, C+++
	XCVI	85	70	92	81	17.9	1.0281	295	0	A+, C+
	XCVII	85	17	21	19	24.6				A+, C+, P+++
	XCVIII	85	32	18	25	99.9				A+, C+, P++
	XCIX	89	33	35	34	31.6	1.0221	378	0.036	A+, C++, P+++
	C	89	59	56	58	13.9	1.0223	273	0	
90–101	CI	91	51	59	55	13.1				P++
	CII	91	40	48	44	21.6				P++
	CIII	101	21	24	23	31.8				A+++, C+, P+

When A = 40, $\overline{UC}$ = 100.2.

When A = 90, $\overline{UC}$ = 54.6 (fig. 1 A).

There is a decline in the fifty-year period of 45.6 per cent; and in each decade, of 4.6 per cent, of normal clearance.

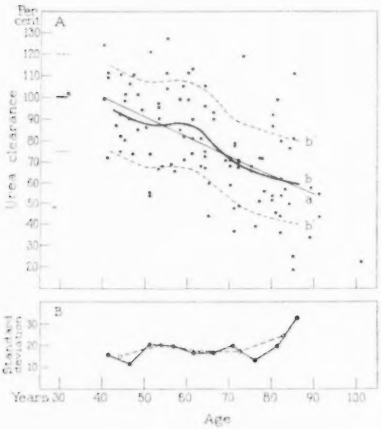

Fig. 1

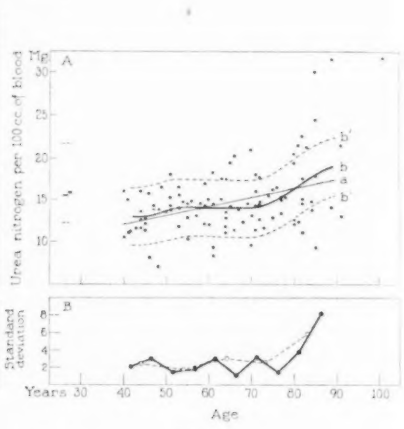

Fig. 2

Fig. 1. Clearance of urea by the kidneys in normal men over 40 years of age, expressed in per cent of average clearance in young normal men (2).

A. Dots (•) represent individual values (averages of two-hour clearance tests); *a* is the straight line regression, 40 to 89 years; *b* is the smoothed regression of five-year means, with limits, *b'* (dashed lines), of standard error of estimate, 40 to 89 years; the asterisk (*) represents the average in young normal men, with limits of variation (short dashed lines) (Möller, McIntosh, Van Slyke, 2).

B. The unbroken line is the standard deviation from means of half-decades of men, aged 40 to 89 years. The broken line is the standard deviation from means of decades of the same men.

Fig. 2. The amount of urea nitrogen in the blood in normal men over 40 years of age.

A. Dots (•) represent individual values; *a* is the straight line regression, 40 to 89 years; *b* is the smoothed regression of five-year means, with limits, *b'* (dashed lines), of standard error of estimate, 40 to 89 years; the asterisk (*) represents the average in young normal men, with limits of variation (short dashed lines) (MacKay and MacKay, 10).

B. The unbroken line is the standard deviation from means of half-decades of men, aged 40 to 89 years. The broken line is the standard deviation from means of decades of the same men.

The coefficient of correlation, -0.54 ± 0.07, represents a highly significant negative relation.

If the results of the series of one hundred and three men, aged 40 to 101 years, are given, the equation is:

$$\overline{UC} = 136.1 - 0.91 \times A$$

When A = 40, $\overline{UC}$ = 99.8.
When A = 99, $\overline{UC}$ = 54.4.
When A = 100, $\overline{UC}$ = 45.3.

The decline is the same as in those aged 40 to 89 years; the coefficient of correlation, -0.59 ± 0.065, is even more significant.

d. From inspection of the trend of the five-year and of the ten-year classes, it is evident that the relation of age and urea clearance is changing and curvilinear rather than uniform and linear. From the 40th to the 55th year, there is a drop. The decline appears then to be arrested for ten years. After the 65th year the decline is again accelerated. It is obvious that the curve undergoes inflections, not apparent in the linear expression, correlated with high significance though it may be.

A curve, obtained by smoothing the five-year means, is utilized in figure 1 A. It has slightly higher measures of correlation and determination and a smaller standard error of estimate. In this curve, 31.0 per cent of the variance in urea clearance is accounted for by variance in years; by the straight line relation 29.5 per cent.

From this experience it is apparent that, as in the case of the basal metabolic rate, a curve representing what is normal can be drawn to express average functions according to age (fig. 4). This normal curve differs from that describing changes in basal metabolic rate in that there (B.M.R.) there are deviations above and below an average normal, both expressive of diseases; where here (UC) high values appear to have less pathological meaning than low ones. Because of this aspect, the curve of probability for the clearance of urea may present modifications.

II. *Amount of urea nitrogen in the blood.* The results are presented in several forms:

a. The mean values in succeeding five-year groups show an increase with age (table 1, fig. 2 A). In the men aged 40 to 44 years, the average was 12.9 mgm. per 100 cc. of blood. A slight increase occurred in the group aged 50 to 54, in those aged 65 to 69, and again in those aged 80 to 84 years. In the men of 85 to 89 years, the average content increased rather sharply to 21.2 mgm. per cent. The individual values ranged from 7.0 (at 48 years) to 31.6 mgm (at 89 years). In a subject of 101 years it was 31.8 mgm. All the subjects with values above 20 mgm. per cent were over 65 years of age. Three, with contents above 25 mgm. per cent, were over 85 years of age (table 1, fig. 2 A).

The standard deviation of individuals from the mean of their age groups showed little difference until the 85th year (fig. 2 B); it was as large in the 45 to 49 as in the 70 to 74 year group. Between 40 and 64 years, the standard deviation was 2.5 mgm., the mean 13.5; between 65 and 89 years, the standard deviation was 4.7 mgm., the mean 16.1. The mean of the series of one hundred men was 14.7, the standard deviation 3.9 mgm. per 100 cc. of blood.

b. Though the mean increased with age, the mode was essentially the same in the various age groups. The median showed a slight increase with age. These facts, apparent in figure 2 A, are shown more clearly if the data of table 1 are arranged in a histogram.

c. The linear equation for the relationship of urea nitrogen of the blood and age is:

$$\overline{BN} = 7.56 + 0.1119 \times A$$

where $\overline{BN}$ = blood urea nitrogen in milligrams per 100 cc. of blood and A = age in years.

When A = 40, $\overline{BN}$ = 12.03.

When A = 90, $\overline{BN}$ = 17.62 (fig. 2 A).

There is an increase for the fifty-year period of 5.59 mgm., or 46 per cent, above the amount at 40 years. The coefficient of correlation, $+0.40 \pm 0.085$, represents highly significant positive correlation.

If the results of the series of one hundred and three men are used, the equation is:

$$\overline{BN} = 6.358 + 0.1322 \times A$$

When A = 40, $\overline{BN}$ = 11.6.

When A = 90, $\overline{BN}$ = 18.3.

When A = 100, $\overline{BN}$ = 19.6.

The coefficient of correlation, $+0.45 \pm 0.08$, represents still higher significant relationship.

d. From inspection of the curves representing the measurements in the five-year and ten-year classes, it is evident that age and the amount of urea nitrogen in the blood have a curvilinear relation. There is a slight rise in the 5th decade, no significant change during the 6th and 7th decades, and a rather sharp increase after the 70th years.

A curve (fig. 2 A) obtained by smoothing the five-year means shows a higher measure of correlation than the straight line. Its index of correlation is $+0.45 \pm 0.08$; its standard error of estimate is 3.5 mgm., instead of 3.8 for the straight line; 21 per cent of its variance is accounted for by the variance in years, whereas in the straight line relation it is only 16 per cent. The standard error of estimate was greatest in the 9th decade; for those 40 to 64 years it was 2.43 mgm.; for those 65 to 89 years it was 4.32 mgm.

From the facts in this study, a table of average values at different ages may be arranged, and a curve of probability may be used to represent the chances of being normal according to deviations from the averages (fig. 4).

III. *The concentrating ability of the kidneys.* The results are presented in several forms:

a. The means of the five-year classes showed a gradual decrease with age, which did not become significant until the 75th year (fig. 3 A); before the 75th year the average specific gravity in these groups was above

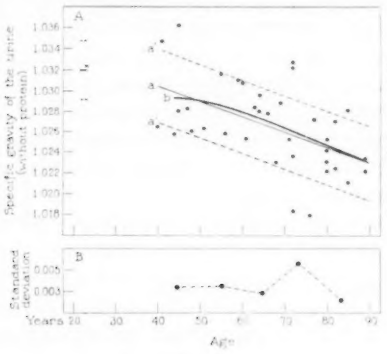

Fig. 3

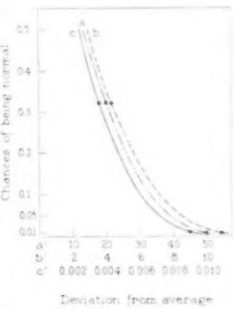

Fig. 4

Fig. 3. Specific gravity of the urine (without protein) in normal men over 40 years of age, derived from the concentration test of Addis and Shevky (14).

A. Dots (•) indicate individual values; *a* is a straight line regression, 40 to 89 years, with limits, *a'* (dashed lines), of standard error of estimate; *b* is the smoothed regression of ten-year means; the asterisk (*) represents the average in young normal individuals, with limits (short dashed lines) of standard deviation (Addis and Shevky, 14).

B. The broken line is the standard deviation from means of decades of men, aged 40 to 89 years.

Fig. 4. Curves describing the chances that certain functions of the kidneys are normal according to deviations from the averages (or "the probability of specified departure" (28)). The ordinates show the chances of being normal, the abscissae the deviations from the averages.

a. Curve of probability for clearance of urea expressed in per cent of mean clearance in young normal men (2), according to deviation, *a'*, from the average.

b. Curve of probability for amount of urea nitrogen in milligrams for 100 cc. of blood, according to deviation, *b'*, from the average.

c. Curve of probability for specific gravity of urine (without protein) according to deviation, *c'*, from the average.

1.026. In the three five-year groups from 75 to 89 years, the means were 1.023, 1.024, and 1.024, respectively. The number of men in each class, however, was small.

From inspection of the averages of the ten-year classes, the decline appears more definite (fig. 1 A). In the 5th decade, the mean value was 1.029; in the 9th, 1.024. In the men of 40 to 64 years, it was 1.029; in

those of 65 to 89 years, it was 1.025. The lowest value in the 40 to 64 year group was 1.025 in a man of 61 years; the highest value was 1.036 in a man of 45 years. In the 65 to 89 year group the lowest value was 1.018 in a man of 76 years, the highest 1.033 in a man of 72 years (table 1). The standard deviation of individuals from a mean of their own age class was slightly greater in the older men (fig. 3 B). The mean of the whole series was 1.0266, with a standard deviation of ± 0.00423.

b. The number in each decade is too few to indicate changes in the mode and the median. However, in separating the cases in twenty-five year groups, before and after the 65th year, it is obvious that the mean, the mode and the median of the 40 to 64 year group are higher than in the older men; this fact, apparent in figure 3 A, is shown more clearly if the data of table 1 are arranged in a histogram.

c. The linear equation for the relationship of the specific gravity and age is:

$$\overline{SP} = 1.03645 - 0.00015 \times A$$

where $\overline{SP}$ = the specific gravity of the urine without protein and A = age in years.

When A = 40, $\overline{SP} = 1.03049$.

When A = 90, $\overline{SP} = 1.02304$ (fig. 3 A).

The decline over the fifty-year period is 0.00745, or 24 per cent of the concentration at age 40. The coefficient of correlation, -0.52 ± 0.12, represents highly significant negative correlation. The standard error of estimate is 0.0036.

d. From inspection of the trends of the five and ten-year classes the specific gravity appears to decrease, slightly from the 40th to 65th year, and more abruptly after the 65th year. The regression, the correlation with age, and the standard error of estimate of the smoothed curve of the ten-year means are similar to those of the straight line (fig. 3 A). It is not possible to decide whether the concentrating activity of the kidneys follows a uniform or an inflected decline. A difference is present, but it is slight in either case. The linear regression may indicate, therefore, the averages of the specific gravity and the chances of being normal according to deviations from the averages (fig. 4).

e. Though the specific gravity, and therefore the concentration of solids in the urine, collected in the second 12 hours of the test, was lower in the elderly men of this series, the urinary volumes were similar. The mean volumes of the decades showed small differences. Individual variations in these men over 40 years of age (table 1) were less marked than in young individuals (17). Higher specific gravities and smaller urinary volumes were more commonly associated. These facts emerge from a study of the data in table 1.

DISCUSSION. I. The action of the kidneys in clearing urea from the blood is by no means uniformly performed in normal individuals; it presents, as do other functions, natural variations. Möller, McIntosh, and Van Slyke (2) remark: "the fact that in a given individual the probable variation of the standard blood urea clearance is 10 per cent, and that the maximum variation is much greater, indicates . . . that other factors in addition to blood urea concentration and urine volume affect urea excretion." "The formulae of the clearance are only expressions of the effects of two factors, blood urea content and urine volume, which are in continual action and appear to be ordinarily of chief importance in regulating the urea output."

It is obvious that in the senescent processes in adult men there is diminution in the removal of urea from the blood by the kidneys. According to Möller, McIntosh, and Van Slyke (2) "decrease in the volume of blood cleared of urea per minute in pathological conditions must be due to one of two causes: either the volume of blood per minute passing through the kidneys is diminished, or the proportion of its urea removed during the passage is less than normal." In normal dogs (23), variations in urea clearance, occurring spontaneously or as the result of changes in protein intake, have been found to be closely correlated with the renal blood flow, variations in the percentage of urea removed from the blood playing a less important part. In glomerulonephritis, damage to renal vessels, especially to the glomerular capillaries, leads no doubt to diminished blood flow through the kidneys; in this, and in the degenerative (nephrosis) form of Bright's disease, decrease in clearance of urea is proportional to glomerular destruction (7).

What senescent changes directly cause the decline in this renal function, we do not know. The decrease of urea clearance with age may depend on decrease of renal blood flow, which, in turn, may be caused either by destruction of glomeruli or by contraction of renal arteries and arterioles. According to the observations of Moore, (24), the total number of glomeruli in the kidney lessens with age; in the 7th decade it was one-third to one-half the count in young adult men. In the rat, the same course of events has been noticed (25, 26, 27). In pathological circumstances, in the arteriosclerotic type of Bright's disease, clearance may decrease markedly without apparent damage in most of the glomeruli; the fall in clearance then appears to be proportional to arteriolar damage (7).

II. Numerous observations on the amount of urea nitrogen in the blood, especially of patients with renal disease, have been reported. Measurements of normal individuals in the later decades have not been systematically collected and analyzed. In the study of MacKay and MacKay (10) on young normal individuals, the highest content of urea nitrogen was 21.7 mgm., the lowest 12.2 mgm.; the mean of the group was 15.5 mgm.

per 100 cc. of blood. They observed a slight increase from the 20th to 34th year, the age limits of their subjects.

In this series, only five men exhibited a content higher than the maximum of 21.7 mgm. per cent observed by MacKay and MacKay; all five were over 80 years of age (fig. 2 A). Significant changes in the clearance of urea were noticed in younger subjects of the present study. MacKay and MacKay observed also (11) that the concentration of urea in the blood of individuals with renal disease does not begin to rise markedly until the renal function falls below half of its original amount; as the amount of functional activity is still further decreased the concentration of urea increases at an accelerated rate. In this study, such acceleration may be noticed after the 75th year, but the loss of renal function as shown by the urea clearance is evident after the 63rd year. Presumably, the gradual increase of urea nitrogen in the blood is preceded by a gradual loss of renal function. In patients with Bright's disease, Möller, McIntosh, and Van Slyke (6) confirmed the results of MacKay and MacKay (11), and observed that unless the excretion rate is also considered, blood urea taken alone may fail to reveal diminution of renal function until it has reached an advanced stage (6, 7).

III. The study of Addis and Shevky (14) contains observations on the concentrating ability of the kidneys in young normal individuals. They made 94 observations of 75 normal persons, most of whom were medical students. The age distribution is not given. The average specific gravity was 1.032, the standard deviation ± 0.00281 or a variability of 8.8 per cent; in ninety-five per cent of the subjects on ordinary diets, the specific gravities were 1.028 or above; all measurements were above 1.026. The odds that any individual of their group under the conditions of the test would give a specific gravity of 1.026 or lower were one in 47; below 1.025, one in 115; below 1.024, one in 323; below 1.023, one in 1,000. In the study of Lashmet and Newburgh (15, 16) all the normal subjects concentrated urine to 1.026 or above. Because of these and other observations, specific gravity below 1.026 in young individuals is considered abnormal.

In this study, in seventeen subjects under the 65th year, only one measurement fell below 1.026, being 1.0253. After the 65th year, in twenty-one subjects, this was the case in fourteen measurements. Among obvious explanations is that the loss of concentrating power is due either to changes in glomerular filtration, in view of the fact that the number of glomeruli is less in older individuals (24), or to decline of tubular reabsorption.

In the men of this study, the specific gravities were not as low as those observed in individuals suffering from renal disease (17).

SUMMARY

Three functions of the kidneys have been measured in normal men over forty years of age to ascertain the average values, the range of variation from the averages, and the rate of change at different ages. Observations were obtained on: I, the ability of the kidneys to remove urea from the blood; II, the amount of urea nitrogen present in the blood; III, the ability of the kidneys to concentrate urine, as exhibited by the specific gravity. The subjects were healthy men; none was an inmate of an institution. They were observed under similar circumstances.

The results have been analysed and compared with the observations already published on young normal individuals.

1. The ability of the kidneys to clear the blood of urea has been measured in one hundred normal men, 40 to 89 years of age, twenty in each decade, and in two men 91 years and one man 101 years of age. This function declined with age.

a. The equation for the regression in the men aged 40 to 89 years is:

$$\overline{UC} = 136.6 - 0.912 \times A$$

where $\overline{UC}$ = urea clearance expressed in per cent of the average clearance in young normal men and A = age in years.

The decline over the fifty-year period is 46 per cent. The correlation, -0.54 ± 0.07, is highly significant.

b. The mean values in succeeding half-decades or decades suggest that the decline does not follow a straight line regression, but an S-shaped curve, being arrested from 55 to 65 years and accelerated from 65 to 75 years.

2. The content of the urea nitrogen in the blood has been measured in the same group of men. The content increased with age.

a. The equation for the increase in the men aged 40 to 89 years is:

$$\overline{BN} = 7.56 + 0.112 \times A$$

where $\overline{BN}$ = urea nitrogen in milligrams per 100 cc. of blood and A = age in years.

The increase over the fifty-year period is 5.59 mgm., or 46 per cent. The correlation, $+0.40 \pm 0.85$, is highly significant.

b. The mean values in succeeding half-decades or decades indicate that the increase does not follow a straight line regression, but an S-shaped curve, the increase being arrested from 53 to 73 years and accelerated thereafter.

3. The concentrating power of the kidneys, as shown by the specific gravity of urinary specimens collected during a period of restricted fluid intake, has been measured in thirty-eight normal subjects from 40 to 89

years of age, seventeen being from 40 to 64 and twenty-one from 65 to 89 years. The specific gravity declined with age.

a. The equation for the regression from 40 to 89 years is:

$$\overline{SP} = 1.036 - 0.00015 \times A$$

where $\overline{SP}$ = non-protein specific gravity and A = age in years.

At age 40, the specific gravity is 1.030; at age 90, 1.023. The decline over the fifty-year period is 0.007. The linear correlation, -0.52 ± 0.12, is highly significant. The linear and curvilinear regressions differ very slightly.

b. The volumes of urine excreted during the period of restricted intake of fluid are similar in the men of different ages of this study. They are similar to the volumes observed in studies on young normal men.

c. The specific gravity does not fall below the lower limit of 1.026, observed in young normal individuals, before the 65th year. In tests on seventeen subjects aged 40 to 64 years, the specific gravity was found to be below 1.026 only once; it was 1.0253 in a man of 61 years. But in twenty-one subjects over 65 years, this occurred fourteen times.

4. Measurements of variation and the average values in the age groups are given. The variation is greater in the elderly.

5. The clearance of urea alters most markedly. The increase of urea nitrogen in the blood occurs slowly and sometimes after decrease of renal function, as shown by the clearance of urea. The decline in specific gravity is gradual; it is less in elderly normal men than in individuals suffering from renal disease.

6. The mechanism of the changes is discussed. The decline in urea clearance may be due to decrease of flow of blood, or of amount of urea removed during the passage of blood, through the kidneys; either effect may be associated with destruction of glomeruli or with contraction of renal arteries and arterioles. The gradual increase of the urea nitrogen follows sometime after the decrease of urea clearance. The loss of concentrating power, resulting in decrease of specific gravity, may be due either to changes in glomerular filtration or to decline of tubular reabsorption.

CONCLUSIONS

1. In normal men over forty years of age, certain functions of the kidneys alter with age. The correlations are highly significant. The average values, the variations from the averages, and the rates of change are given in graphs and equations.

2. The clearance of urea, expressed in per cent of average clearance in young normal men, declines from 100 per cent at 40 years to 55 per cent at 89 years.

3. The content of urea nitrogen in the blood, in the same men, increases

from 12.03 mgm. per 100 cc. of blood at 40 years to 17.62 mgm. at 89 years.

4. The concentrating ability of the kidneys, as shown by the urinary specific gravity in thirty-eight men, declines from 1.030 at 40 years to 1.023 at 89 years.

5. The mechanism of the changes deserves but has received no study.

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THE RATE OF REMOVAL OF HEMOGLOBIN FROM THE CIRCULATION AND ITS RENAL THRESHOLD IN HUMAN BEINGS

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Hemoglobinemia frequently occurs in disease. It seems strange that there is no precise knowledge as to how rapidly hemoglobin is removed from the blood or how much is necessary to produce hemoglobinuria in human beings.

Whipple and collaborators (2, 3) determined several of the factors for dogs. Sellards and Minot (1) showed that intravenous injection in human beings of moderate amounts of human hemoglobin is innocuous. They indicated in terms of red blood cells an approximate amount needed to produce hemoglobinuria. They did not measure the plasma concentrations. In the numerous studies of acute hemolytic processes there have been no observations on the amount or duration of the hemoglobinemia.

In this paper we shall present studies of the results of one or more intravenous injections of sterile, stroma-free, human hemoglobin into twenty normal persons. For safety the amount of hemoglobin injected was kept relatively small—usually 4 to 8 grams—and generally just below or just above the point at which hemoglobinuria was to be expected. This amount represented the hemoglobin of 20 to 50 cc. of blood.

METHODS. *Preparation of hemoglobin.* Blood was collected as for transfusion with sodium citrate. In the early cases the subject's own blood was used. Subsequently, when we were certain that all the stromata had been removed, other available blood was used without regard to blood groups.

The well-washed cells were laked with four volumes of sterile distilled water and kept at 37°C. for one to two hours with frequent shaking. One-twentieth the volume of 18 per cent saline solution was then added and the clear dark red solution became opaque as the heretofore invisible stromata became visible. This solution was then centrifuged rapidly for one hour when the clear supernatant fluid was removed.

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This supernatant hemoglobin solution unfortunately always showed, on addition of a little hypertonic saline solution, the presence of some stromata. These are believed to be the chief injurious element in the injection of laked blood (4). These are most readily removed by filtration of the solution through a Seitz filter, which insures sterility.

The hemoglobin solution was usually 100 to 200 cc. For safety five to twelve minutes were taken for the injection. This may have caused slight transient variations (5) in the initial concentration of hemoglobin in the plasma. The hemoglobin content of the injection solution was determined by the method of Robscheit-Robbins (6).

Collection of blood samples. Because we were measuring hemoglobin in the plasma it was essential that there should be no hemolysis in the collection of blood specimens. Hemoglobin free control specimens of plasma were invariably obtained when $\frac{1}{10}$ volume of 3 per cent sodium citrate solution was first drawn into the syringe and at once mixed with the blood. After ten minutes, centrifuging the supernatant solution was measured. To allow for the dilution with citrate, the quantitative hemoglobin estimations subsequently obtained were corrected by the factor

$$\frac{\text{cc. supernatant fluid}}{\text{cc. supernatant fluid} - \text{cc. citrate solution used}}$$

Specimens were collected six : ' twelve minutes after the injection, and at approximately two or three hour intervals thereafter, until the plasma became colorless or nearly so.

Estimation of hemoglobin in plasma and in urine. The plasma specimens showed successive decreases in color indicating approximately the concentrations of hemoglobin. Direct comparisons (2) with the injected solution were unsatisfactory because human plasma is not colorless like dog plasma, and the color is variable.

Bing and Baker's (8) modification of Wu's benzidine method checked satisfactorily (9) after two sources of error were eliminated. First, benzidine (10) that did not develop some color in the blanks could not be purchased but was obtained by dissolving C. P. benzidine in hot benzene, precipitating the filtered solution with petroleum ether and drying. The second error was in the standard boric acid solution of blood. It required frequent restandardization and was therefore dropped. Instead the hemoglobin solution used for injection in each case served as a standard: its hemoglobin content (by the Robscheit-Robbins method) was already accurately known. It was simply diluted $\frac{1}{100}$ and carried through the same benzidine procedure as the plasma specimens.

The first two plasma specimens after each injection always contained a large amount of hemoglobin and were diluted $\frac{1}{100}$ or $\frac{1}{50}$ with distilled

water. Subsequent specimens were diluted less; the last was diluted $\frac{1}{5}$. These dilutions were essential to bring the color developed closer to the standard for accurate colorimetry. A $\frac{1}{5}$ minimal dilution of the plasma was necessary to prevent turbidity caused by precipitation of the protein by the acetic acid and alcohol of the benzidine reagent.

The urines were diluted according to the intensity of the hemoglobinuria, usually $\frac{1}{25}$, and treated in the same way as the plasma specimens.

Collection of urine specimens. To minimize the possibility of kidney damage (11) the patients were given 2 grams of sodium bicarbonate (12) with 250 cc. of water before the injection and considerable quantities of water, about 200 cc. hourly for the first three or four hours. This undoubtedly caused some change in the plasma volume, but as diuresis paralleled absorption the alteration was probably not very significant (13). Urines were collected every half or one hour.

Plasma volume determinations. In the first cases injected the plasma volume was merely estimated by the method of Rowntree and Brown (14) from the hematocrit, weight and sex. In the subsequent cases it was measured by the Congo Red method of Keith and Rowntree. Specimens collected 7 or 8 minutes instead of 6 minutes after the injection of the Congo Red made no great difference in our values (which were within the range found by Gibson and Evans (15), males 35.4 to 58.2, females 26.9 to 52.2 cc. per kgm.).

Estimation of plasma bilirubin. The method of Thanhauser and Anderson (16) was used.

Experimental observations. A. *Bilirubinemia.* At first we were surprised that there was no or a barely detectable but not measurable increase in the plasma bilirubin (17). The following calculation will show that this finding was to be expected. According to Abderhalden (18) hemoglobin on conversion yields 4 per cent of hematin and hematin is convertible into an equal amount of bilirubin. When 5 grams of hemoglobin (an average dose in this series) was injected, a maximum of 200 mgm. of bilirubin could be formed. The normal human liver excretes bilirubin so rapidly (19) that such an amount especially if gradually formed and diluted in the entire plasma volume, would not be detected.

B. *Initial level of hemoglobin in plasma.* In the seventeen patients in whom the plasma volume was measured the initial level of plasma hemoglobin to be expected was calculated from plasma volume and amount of hemoglobin given (with a correction for the volume of injection solution). These results are tabulated in parentheses next to value for actual initial level (table 1, column D). They agree fairly well with the initial level actually observed except in two persons (cases 36 and 54, A, B, C). The approximate agreement between observed and calculated initial levels indicates that injected hemoglobin is distributed uniformly

throughout the plasma without immediate removal or conversion. It thus might be used as a dye for the determination of plasma volume (20).

C. *The rate of removal of hemoglobin from the plasma.* The rate of removal of hemoglobin from the plasma can best be studied by plotting the results on graphs (see fig. 1).

TABLE 1

Cases arranged in order of increasing dosage in milligrams per 100 cc.

A	B	C	D	E	F	G	H
CASE NO.	SEX	DOSAGE	ACTUAL INITIAL LEVEL OF PLASMA HEMOGLOBIN MGM. PER 100 CC. PREDICTED LEVEL IN ( )	DOSE OF HEMOGLOBIN	HEMOGLOBIN IN URINE	DURATION OF HEMOGLOBINURIA	PLASMA LEVEL AT END OF HEMOGLOBINURIA
		mgm. per kgm.		*grams*	*grams*	*hours*	*mgm. per 100 cc.*
44B	M	52	114 (130)	3.78	0		
43A	M	57	105 (131)	3.47	0		
3	M	66	288	5.06	0		
29	M	66	137 (134)	4.0	0		
11	F	67	175	5.34	0		
52	F	72	146 (143)	4.57	0.007	2	100
15	F	75	177	4.5	0.28	3	125
47	F	77	203 (203)	5.38	0.10	2	140
53	F	80	216 (215)	6.94	0.3	7	40
5	M	78	202	5.88	0		
17	M	79	240	4.5	0		
54A	M	81	96 (155)	4.1	0		
4	M	88	240	4.0	0		
38	M	88	130 (144)	5.3	0		
12	M	92	285	6.56	0.3	6	100
44A	M	95	230 (231)	6.95	0.4	5½	50
54B	M	98	110 (180)	5.48	0.18	3¼	50
54C	M	98	110 (180)	5.48	0.25	5	30
36	M	100	142 (182)	6.0	0.93	4	85
51	M	100	234 (220)	6.2	0.5	4	50
48A	M	102	246 (215)	6.15	0		
50	M	104	247 (192)	7.3	0.6	9 (Bladder lag)	
9	M	106	260	5.8	0		
43B	M	114	255 (248)	6.95	0.5	4	50
48B	M	237	322 (280)	8.25	0.4	3¾	140

There are two types of curves. One has a uniformly gradual slope; less than 40 per cent of the hemoglobin was removed within three hours after injection. This occurred with low initial levels (100 to 185 mgm. per cent) and small doses (average 74 mgm. per kgm.) (fig. 1, cases 36, 44B, 43A). The other type of curve occurred in cases that had received larger doses and consequently had higher initial plasma levels. This

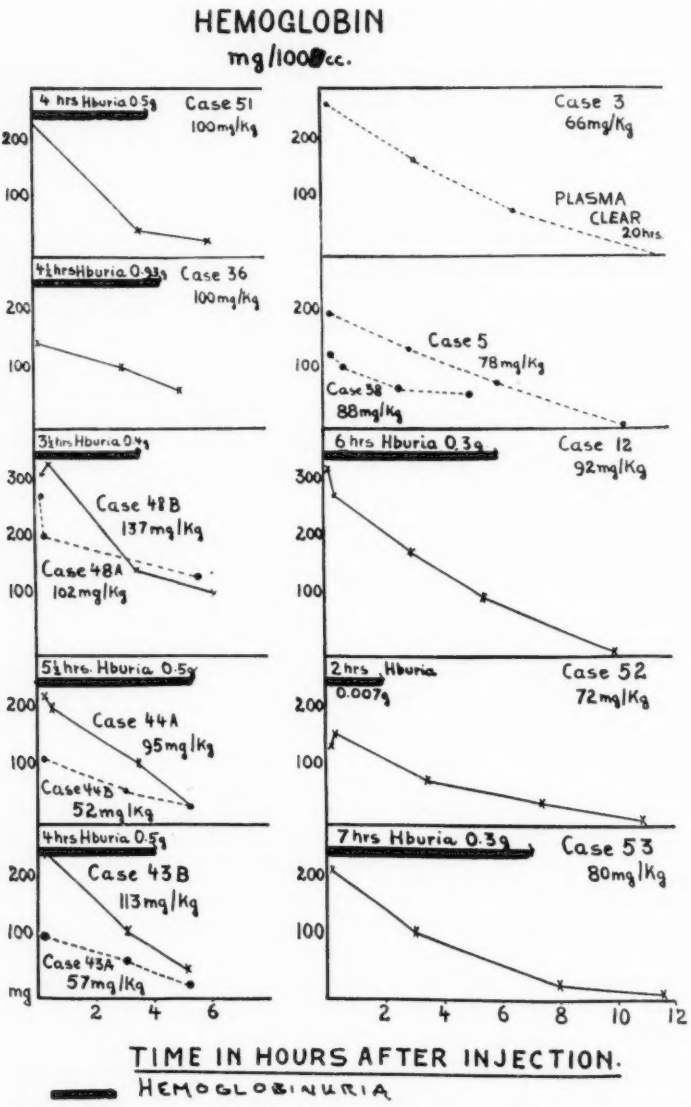

Fig. 1. Rate of removal of hemoglobin from the plasma. Each curve represents one injection. Where two curves appear (*A* and *B*) they represent two injections of the same individual with different doses. Dotted lines indicate injections not followed by hemoglobinuria. Solid lines represent injections which produced hemoglobinuria. Black area represents hemoglobinuria.

curve fell more sharply in the first three hours, indicating 50 per cent or more removal of hemoglobin, and then gradually approximated the other type of curve in the later hours. In these cases the initial levels varied from 200 to 300 mgm. per cent and the average dose was 122 mgm. per kgm. (fig. 1, cases 51, 48B, 44A, 43B).

Hemoglobinemia was moderately persistent; the low plasma level of 50 mgm. per cent was not reached until at least five hours after injection (except case 51, in three hours). Seven cases followed longer took over ten but less than twenty hours for the plasma to become hemoglobin free.

From the curves with precipitate declines in the early hours we can estimate that only a small part of the hemoglobin removed from the blood is accounted for by that excreted in the urine (see fig. 1). Thus, in case 43B, about 4.2 grams were removed from the blood stream in three hours but only 0.49 gram appeared in the urine. In the same patient (case 43A) following injection of a smaller dose, there was no hemoglobinuria and the flat type of curve was found, only 1.4 grams hemoglobin being removed from the plasma in the same interval. Hemoglobinuria per se evidently does not account for the early rapid removal following large doses.

In all cases the amount of hemoglobin lost in the urine was relatively scant, usually less than 7 per cent of the amount injected; the hemoglobinuria seldom lasted more than six hours and was uniformly symptomless.

It is important to notice (table 1, columns G and H) that in cases in which hemoglobinuria occurred at all it usually continued until a time when the plasma level was relatively low,—far below the initial level in most of those cases that showed no hemoglobinuria. Allowance must be made for the fact that urines were collected only every half or one hour; but after correction has been made for this the observation is still valid. When the amount of hemoglobin lost in successive specimens of urine was measured it was found that usually over half the total was lost in the first hour. It is possible that the hemoglobin appearing in the urine after three or more hours had been excreted into the tubules previously, and its appearance in small amounts in the later hours represented merely mechanical washing out of the urinary tract. This would explain the apparent inconsistency of hemoglobinuria continuing at a time when the blood level of hemoglobin was very low.

D. *Occurrence of hemoglobinuria.* Hemoglobinuria occurred in thirteen of the twenty-five injections. Three variables in our experiments might have influenced it: 1, speed of injection; 2, plasma volume; 3, total dose.

1. The injections varied in duration from 5 to 25 minutes. The following experiment (table 2) was done to determine whether this affected the result.

A normal male, at weekly intervals, was tested with: A. Rapid injection of a dose just insufficient to cause hemoglobinuria. B. Rapid injection

of a dose just sufficient to cause hemoglobinuria. C. Slow injection of a dose just sufficient to cause hemoglobinuria. The results show that the variations in the rate of injections in our experiments played no rôle in determining the occurrence of hemoglobinuria. In view of the relatively slow removal of hemoglobin from the plasma this conforms to expectation.

2. Plasma volume is important because the initial plasma hemoglobin is inversely proportional to it. When we began our work we expected that hemoglobin, like many other substances excreted by the kidneys, would show some definite plasma concentration beyond which it would appear in the urine (21). This turned out not to be the case (table 1, columns D and F). If there is a renal threshold in the sense of a definite plasma concentration needed for hemoglobinuria in human beings it varies from individual to individual within very wide limits. Thus, of the thirteen cases showing hemoglobinuria eight had fairly high initial levels of hemoglobin in the plasma (200 mgm. per 100 cc. or over) but

TABLE 2

Effect of variation of rate of injection

(Three injections in same patient at one week intervals)

CASE NO.	DOSE	TIME REQUIRED FOR INJECTION	INITIAL LEVEL	HEMOGLOBINURIA
	mgm. per kgm.		*mgm. per 100 cc.*	*grams*
54 A	81	25 seconds	96	0
54 B	98	55 seconds	110	0.18
54 C	98	11 minutes	110	0.25

five had low initial levels. On the other hand there were six cases with high plasma levels (200 to 288 mgm.) which did not have hemoglobinuria.

Nor were the amounts of hemoglobin lost in the urine related to the level of plasma hemoglobin reached. Case 48B with the highest plasma level—322 mgm. per 100 cc.—lost only 0.4 gram, while case 36, with the low level of 142 mgm. lost 0.93 gram.

3. Since the plasma concentration of hemoglobin is not the controlling factor in hemoglobinuria, is the occurrence of hemoglobinuria related to dosage at all? Obviously it is. If table 1 is studied it will be noticed that all the cases that show hemoglobinuria fall into two groups: 1, the cases which show hemoglobinuria at a low dosage (72 to 80 mgm. per kgm.); these are all women; 2, the cases which show it only at a dosage of 92 mgm. or higher per kgm.; these are all men. No male showed hemoglobinuria with a dose of less than 92 mgm. per kgm. However, not all the males receiving the large dose developed hemoglobinuria: two cases with 102 and 106 mgm. per kgm. failed to do so.

There is then a threshold in terms of body weight and it is lower for women than for men.

Discussion. The degree of correspondence between the observed initial levels of hemoglobin in the plasma and the expected levels offers a check on the accuracy of the various methods. As was anticipated the initial level varied directly with the dose in terms of plasma. When, however, the dose is expressed in terms of body weight the relationship to plasma level is irregular insofar as the relationship of plasma volume to body weight is irregular.

The two types of curves of removal of hemoglobin from the plasma may be important for understanding the factors responsible for hemoglobinuria. When the plasma hemoglobin level does not exceed some rather variable limit, about 150 mgm. per cent, there is a fairly uniform and gradual removal from the plasma of the injected hemoglobin: we may assume that the reticulo-endothelial system gradually disposes of it (17). When, however, this limit is exceeded there is a precipitate drop in the plasma level of hemoglobin in the first three to five hours, and hemoglobinuria generally occurs.

It is possible that beyond this limit some special mechanism enters: if the amount of hemoglobin lost in the urine were enough to account for the increased rate of removal from the plasma the explanation would be simple. But this is not the case; in some instances indeed there is no hemoglobinuria at all despite the rapid initial removal.

An explanation might be offered based on the theory of Whipple and his co-workers. Apparently above the assumed variable limit, hemoglobin passes through the glomerular membrane and is absorbed and retained by the cells of the tubules thereby rapidly reducing the level of plasma hemoglobin. This limit, then, corresponds to Lichty, Havill and Whipple's "glomerular threshold." Only when the speed of filtration through the glomeruli is so rapid that the tubular epithelium can not absorb all the filtered hemoglobin does some escape absorption and hemoglobinuria occurs. When sufficient hemoglobin has been absorbed by the tubules or excreted to bring the plasma concentration below the assumed "glomerular threshold," filtration ceases and the usual gradual removal by the reticulo-endothelial system ensues, as indicated by the flattening out of these curves about three to five hours after injections.

Hemoglobinuria will occur only when filtration exceeds the capacity of the tubules to absorb hemoglobin. Thus, although most of the cases with precipitate declines in the early hours showed hemoglobinuria, there are two cases, nos. 3 and 48A, where early moderately rapid removal was not accompanied by hemoglobinuria. In these cases presumably tubular absorption was at least equal to glomerular filtration so that no hemoglobin escaped into the urine. This would account for the large amount of hemoglobin removed from the plasma in the first few hours after injection. Thus, glomerular filtration is explained as a threshold phenomenon but hemoglobinuria is an expression of the inability of the

tubules to absorb and retain all the hemoglobin that had passed the glomeruli.

SUMMARY

The primary object of the work was to establish normal standards for human beings. Human hemoglobin was prepared entirely free from stromata. Twenty normal persons received intravenous injections of varying amounts (3.47 to 8.25 grams) without regard to blood grouping. There were no untoward symptoms.

A method was devised for the collection of blood plasma with absolutely no manipulative hemolysis. No bilirubinemia was produced. This is explicable by the small amount of bilirubin formed and the perfect liver function.

The initial concentrations of hemoglobin in the plasma were dependent directly on the plasma volume. The rates of removal of hemoglobin from the plasma after large and small doses of hemoglobin were different. After large doses it was very rapid during the first three hours, suggesting some special mechanism. The amount of urinary hemoglobin was insufficient to explain this. As compared with many other substances the rate of removal of hemoglobin from the plasma is slow: it required ten or more hours for the plasma to be cleared.

Hemoglobinuria occurred in thirteen instances. In terms of plasma concentration the renal threshold for human hemoglobin was found to be very variable.

In terms of dose of hemoglobin per kilogram of body weight a more constant threshold for hemoglobin was found. Unexpectedly it was different for the two sexes. In women it was 72 mgm., in men 92 mgm. per kilo.

As an explanation of the mechanism of hemoglobinuria the "glomerular threshold" concept of Whipple is tentatively accepted: above some as yet not precisely measurable plasma concentration, hemoglobin is filtered through the glomeruli; it is rapidly absorbed by the renal tubular epithelium. When the rate of filtration exceeds the absorptive capacity of the tubules, hemoglobin is found in the urine.

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THE EFFECT OF DIFFERENT PER CENTS OF PROTEIN IN THE DIET IN SUCCESSIVE GENERATIONS[1]

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The exact amount of protein in the diet which would result in best growth, greatest resistance to ailments or disease, and produce the greatest efficiency of the physiological functions of an animal over an extended life span has not been definitely agreed upon by physiologists and dietitians. It was with the hope of obtaining additional information which might assist in reaching a more definite conclusion that this research was undertaken.

The albino rat was chosen for the experimental animal because its diet could be absolutely controlled, its environment regulated and its span of life is relatively short as compared to man. Any physiological principles established in one animal may be utilized in another species.

The results of five different per cents of protein in the diet on the first matings and on bachelor and virgin rats have been previously published (Slonaker, 1931a, b, c, d, e, f, g and 1935). The purpose of this paper is to present the results of six succeeding generations of rats fed continuously on the same diets as their ancestors.

A detailed description of the diet used for each of the five groups of animals has been given (Slonaker, 1931a). The range in the per cent of protein was from 10.3 to 26.3 per cent. These per cents are well below and above the amount of protein usually consumed by man. Each of the diets was a carefully prepared synthetic one and contained all the known amino acids and vitamins. The diets were designated I, II, III, IV, and V and contained the following per cents of protein, fats and carbohydrates respectively: I, 10.3, 12.2, 77.5; II, 14.2, 14.2, 71.6; III, 18.2, 15.9, 65.9; IV, 22.2, 17.8, 60.0; V, 26.3, 19.7, 54.0.

The animals comprising these successive generations were all kept in

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the same room and under the same environmental conditions as their ancestors. The only variable factor which did equally affect all groups was the diet. Any variations in the results may therefore be reasonably attributed to the differences in the diets on which they were reared. The animals used as matings for the succeeding generations in each group were selected from the preceding generation of that group. Strong, healthy animals were chosen at random and no attempt was made to select them from large litters. When one of a pair died it was replaced by a younger animal to determine the sexual span of the remaining rat. When a pair failed to reproduce each sex was tested with new animals for sterility. Due to a lack of sufficient equipment the spontaneous activity of these succeeding generations was not determined. After the litters were weaned the sexes were segregated and fed the same diet as that of their parents until they had approximated their maximum weight. The life span of the offspring could not be determined due to lack of room and cages. Only the mated pairs were permitted to live their natural life span.

The object of this experiment was not only to determine the effect of the different per cents of protein in the diet, but also to see what would be the effect of these diets when continued through several generations. The experiment extended through six generations and required almost eight years.

Due to restrictions on the length of papers by the editorial staff the presentation of the results of this research will have to assume little more than an abstract form. Plans are being made to publish in detail the complete results of the experiment in the University Series of Stanford University.

Growth and development. The rate of development of the young can be determined by the age at the eruption of the incisor teeth, the opening of the eyes, the disappearance of the vaginal membrane and by the increase in weight.

Eruption of incisor teeth. This was determined by gently rubbing the gums with a glass or metal rod. In all cases the upper incisors erupted about one day earlier than the lower. Variations in the average age at eruption was noted in the generations of each diet group, but these variations were not uniform nor did they suggest a trend as to cause and effect. When all the observations in each diet group were compared we found for the upper incisors that there was a progressive increase in the average age from group I (8.30 days) to group V (8.61 days). With the exception of group III the same relation obtained for the lower incisors, the average ages being group I, 9.43 days and group V, 9.77 days. In general we may conclude that within our limits an increase of protein in the diet delays the eruption of the incisor teeth.

Opening of the eyes. Our criterion for this event was when the lids

could be gently parted so as to expose the eye. The results showed, with few exceptions, a general increase in the age from the first to the last generation. We have no explanation for these exceptions. What effect further generations would have had is problematical, but the results seem to indicate that the limit of retardation had about been reached.

The average results of all generations in each diet group showed, with one exception, that with each increase of protein in the diet there resulted an earlier opening of the eyes. The order of the groups from earliest to latest was: V, III, IV, II and I. The only explanation we can offer for the shifting of groups III and IV was due to an unwise discarding of offspring in group IV thus leaving too few strong animals to use for matings which produced a general reduction of vitality. In our opinion this accounts largely for the erratic behavior of this group throughout the experiment.

Opening of vagina. A wide range in age (23 to 154 days) when the vaginal membrane disappeared was noted. The two earliest ages, 23 and 32 days, may have been due to a congenital absence of the membrane. Mating tests were not made to determine if these animals were sexually mature. The results of succeeding generations in each group were quite variable and showed no definite trend. We therefore conclude that this number of generations had little or no effect in modifying the age at which the vagina opened.

To show the effect of the different diets the results of all generations in each group were considered. This showed that the diets of groups III and II were most efficient in advancing sexual maturity, as measured by the opening of the vagina. The order of the groups and the average ages were: III, 51.1, II, 55.2; V, 56.8; IV, 57.8; I, 68.7 days respectively.

Growth. The increase in weight will be presented under two headings: —mated pairs and the offspring.

Mated pairs. These represent the animals selected from the offspring of the preceding generation matings in their respective groups for the succeeding generation. The average maximum weights and the ages at which these were attained were quite variable in the generations of each group. No definite trend of effect of this number of generations was noted in either sex.

To show the effect of the diets the average maximum weights of all mated animals in each group were compared. Both sexes of group II attained the greatest weight. The order of the groups from greatest to least average maximum weight and the ages at which these were reached were for the males: II, 314, 543; V, 310, 486; III, 297, 518; I, 286, 582; and IV, 274, 489 grams and days. The order for the females was: II, 264, 572; III, 245, 580; V, 244, 513; IV, 237, 532 and I, 231, 584 grams and days respectively. The lack of conformity of ages and that the weight

of a female is modified by breeding may partly account for the difference in the order of the two sexes. The results further showed that not only was the age of maximum weight in both sexes greatest in group I, but, with one slight exception of eight days, there was a progressive decrease in age with each increase of protein in the diet.

Stature. In order to determine whether these diets had any effect on the stature of the animals which could be correlated with maximum weight careful measurements were made of body and tail lengths when they were autopsied. The length of the tail is not a reliable measure of stature. There is a marked tendency for rats to lose portions of the tip of the tail during life which made these results so variable that they were not used. Before taking the body length of old rats, which have a great tendency toward arched back, the spine was straightened out to normal and the length in millimeters from tip of nose to base of tail taken.

Since the results of succeeding generations were quite variable and failed to show any tendency in any way, they will not be given.

When the average of all the animals in each group was considered we found that both sexes of group V had the greatest body length. The average body lengths of the males and females from greatest to least was: V, 192 and 181; II, 191 and 178; III, 186 and 177; IV, 186 and 176; I, 183 and 174 mm. respectively. These figures represent the averages of a number of each sex ranging from 71 to 115 in the different groups. They show that 10.3 per cent of protein in the diet tends to hinder growth in stature while the higher per cents were adequate.

Growth of offspring. The young were weighed daily from birth until weaned. The weights of each sex were recorded separately. After weaning and segregation of the sexes the weights were taken each three or four days until approximately six weeks of age. After this they were weighed the first of each month until they had reached their maximum growth or had to be discarded to make space for later litters. The average weights of only four ages will be compared: birth, 25, 70 and 190 days. These will be sufficient to illustrate the general result.

There was a slight tendency toward a reduction of weight at birth in the later generations in all the groups. With but three exceptions in the sixty possibilities, the weight at birth of the first generation in each diet group equaled or surpassed that of any of the other generations. These exceptions were the males of the second generation in groups I, II and V. The results indicate that this reduction had practically reached its limit and that future generations would have little or no additional effect.

At the age of 25 days both sexes of the later generations in nearly all cases were noticeably heavier than the first generation in all the groups. Though the average weights were variable they do show that six generations had no dilatory effect on growth up to this age.

With but two exceptions, at the age of 70 days, the results further showed that in both sexes the average weights in each of the succeeding generations were heavier than in the first generation. The differences in the average weights of the first and last generations in each of the diet groups for the males and females were: I, 23.8 and 15.2; II, 26.8 and 20.4; III, 23.8 and 6.0; IV, 10.0 and 6.8; V, 25.5 and 10.5 grams respectively. These results show that up to this age these diets were more than adequate for normal growth.

At the age of 190 days the average weights of each sex showed this same tendency of increased weight in later generations. There were, however, more exceptions and a more nearly uniform average weight.

These results show that not only were these diets adequate for normal growth, but that the later generations were able to utilize their food energy more efficiently.

To show the effect of the different diets we have compared the average weights of the offspring of all the generations in each group at the ages of: birth, 25, 70 and 190 days.

At birth both sexes of group III surpassed all other groups in weight. The males showed the following order and average weights from greatest to least: III, 5.37; IV, 5.29; II, 5.22; V, 5.19; I, 5.15 grams respectively. The order and weight of the females was: III, 5.14; IV, II and V each 4.98; I, 4.97 grams respectively. This shows that during prenatal development the diet containing 18.2 per cent protein was most favorable. The number of animals involved in computing these average weights ranged from 1073 in group I to 299 in group IV.

At 25 days the order of average weights in both sexes was the same, except one slight change, from that at birth. The order from greatest to least and the average weights for each sex were: V, 32.5 and 31.0; III, 30.8 and 30.8; IV, 28.4 and 26.5; II, 26.7 and 25.2; I, 20.8 and 20.2 grams for males and females respectively. The lowered vitality of group IV, as explained above, may account for it not being second in order. The number of animals involved in these average weights ranged from 1047 in group II to 280 in group IV.

The groups presented the same order of average weights at 70 days as found at 25 days of age. The number of animals from which these averages were made ranged from 770 in group I to 170 in group IV. The average weights for the males and females were: V, 128 and 108.5; III, 112 and 102.6; IV, 105.6 and 95.5; II, 99.2 and 89.2; I, 72.4 and 69.2 grams for the males and females respectively. The average percentage gain from the 25th to the 70th day in each of these cases was: V, 293 and 250; III, 263 and 233; IV, 272 and 260; II, 271 and 254; I, 248 and 243 per cent for the males and females respectively. The order of these percentage gains fails to conform with that of weight, but we fail to see any significance.

At the age of 190 days the order of the groups from largest to smallest average weight changed slightly from that of 70 days and the females of group IV failed to conform with the males. The number of animals involved in these average weights ranged from 339 in group I to 84 in group IV. The order of the groups with the weights of the males was: III, 217; II, 214; IV, 208; V, 207; I, 177. For the females: III, 182; V, 182; IV, 164; II, 162; and group I, 138 grams. The percentage gains from 25 to 190 days, from greatest to least was for the males: I, 772; II, 702; IV, 632; III, 605; and V, 537 per cent. For the females it was: I, 582; II, 542; IV, 518; III, 490; and V, 487 per cent. The percentage gain from 70 to 190 days from greatest to least was for the males: I, 146; II, 116; IV, 97; III, 94; and V, 53 per cent. For the females it was: I, 101; II, 82; III, 77; IV, 72; and V, 68 per cent. The slight shifting in position in groups III and IV in the two sexes is not significant. These figures show that in general the groups receiving a richer supply of protein had the most rapid increase in weight up to the 70th day of age after which there was a relative decrease in growth from group I to group V up to the 190th day. A relatively few of the offspring could be kept beyond 250 days of age. Their maximum weight was therefore not ascertained. But at 250 days, with the numbers of each sex in the groups varying between 128 and 298, the average weights had the same order of value as given for 190 days.

Fecundity. The animals used in mating for each succeeding generation were selected from the preceding generation in the same diet group. With the exception of the first generation, which had eighteen pairs in each group, the following generations had approximately twelve pairs in each group. A greater number could not be used because of the limited number of cages.

Sterility. Some of the matings were nonproductive. In such cases tests were made with each animal to see which sex was sterile. In some cases both proved sterile. In general there was a noticeable tendency in all groups toward a reduction in sterility with succeeding generations. This was especially noted in groups III, IV and V. The improvement, however, was not uniform. The per cents of total sterility for the first and last generations of these three groups were: III, 27.8 and 00.0; IV, 41.7 and 20.0; V, 69.5 and 7.1 respectively. No more definite statement, however, is warranted.

To get the effect on sterility of the different diets the results of all the generations in each diet group were combined and showed the following total per cents of sterile animals in each group: I, 5.84; II, 2.65; III, 22.94; IV, 39.16 and V, 27.49. In each of the groups, with the exception of groups I and III, the males showed a greater per cent sterile than the females. The per cents of sterile males and females in each of the diet

groups were: I, 5.25 and 6.40; II, 2.70 and 2.59; III, 22.89 and 23.00; IV, 40.00 and 38.56; and V, 33.77 and 21.69 per cent for males and females respectively. The order of sterility in the groups from least to greatest, for both males and females was II, I, III, V, IV. These results show that protein in the diet equal to or in excess of 18.2 per cent greatly interferes with reproduction by increasing sterility.

Reproduction. When the number of days intervening between the age at mating and the age at birth of first litter was considered we found no consistant or uniform results in succeeding generations. With the exception of group I the first generation had the greatest number of days intervening between the average age of mating and average age at birth of the first litter. This was partly due to the early age at mating of all the generation I animals. All were mated at ages ranging from 20 to 49 days which was some time before sexual maturity. Regardless of this advantage there were only two groups (I and II) in which the average age at birth of the first litter in generation I was younger than in later generations. The average range in this interval in the generations of each group was: I, 26 to 92; II, 40 to 102; III, 38 to 197; IV, 24 to 299; V, 48 to 328 days. There was a general tendency toward a shortening of this interval with succeeding generations, but it was not uniformally progressive. No definite conclusions are warranted.

When the results of all generations of each group are brought together we find the average intervals for the males and females were: group I, 66 and 65; II, 69 and 67; III, 93 and 91; IV, 124 and 131; and V, 84 and 84 days for males and females respectively. This shows that the higher per cents of protein in the diet hindered reproduction by delaying the birth of the first litter.

Reproductive span. The days intervening between the average age at birth of the first and last litters was used as representing the reproductive span. Strictly speaking this interval should be increased by approximately fifty days to include the gestation and nursing periods. The average reproductive span in the different generations in each group showed a wide range and no definite indication. The average ranges of the males and females in the five diet groups were: I, 169 to 589 and 161 to 373; II, 214 to 388 and 142 to 351; III, 178 to 314 and 176 to 281; IV, 46 to 185 and 45 to 195; and V, 123 to 357 and 29 to 357 days respectively. When the average reproductive span of the fertile animals of all generations in each diet group were considered the following average reproductive spans for the males and the females were obtained: I, 292 and 221; II, 228 and 239; III, 211 and 207; IV, 145 and 134; and V, 216 and 215 days respectively. These results show a marked tendency toward a shortening of the breeding period as the diet became richer in protein. The order of the groups from longest span to shortest for the males was: I, II, V,

III and IV. For the females it was II, I, V, III and IV. The only difference in the order of the two sexes was in groups I and II.

Loss of weight of mothers during nursing. It is well known that during lactation the mother loses weight and that this loss is in proportion to the number of young nursed. We have therefore determined not only the average per cent lost but also the average loss in grams for each young nursed. The succeeding generations in each group showed a marked, though not uniform, reduction in the per cent of weight lost and the grams lost for each young nursed from the first to the last generation. The per cents lost for the first and last generations in each diet group were: I, 14.90 and 0.55; II, 9.34 and 0.00; III, 7.88 and 3.44; IV, 6.88 and 1.55; and V, 6.08 and 3.49 per cent respectively. The average grams lost in the first and last generations for each young nursed were: I, 5.60 and 0.26; II, 3.78 and 0.00; III, 4.32 and 1.74; IV, 3.49 and 0.71; and V, 2.92 and 2.00 respectively. The average sizes of the litters nursed in the first and last generations were: I, 5.54 and 3.81; II, 5.30 and 3.75; III, 4.40 and 4.03; IV, 4.86 and 4.42; and V, 4.46 and 4.00 respectively. The reduction in the size of the litter nursed played an important part in the weight lost by the mother. The number of litters from which the above average results were obtained were: I, 63 and 21; II, 76 and 24; III, 30 and 32; IV, 22 and 26; V, 15 and 19 litters respectively. The age of the mother while nursing may possibly have had some influence. The average ages of the mothers at the birth of the litters in the first and last generations were: I, 304 and 279; II, 295 and 301; III, 344 and 203; IV, 420 and 274; V, 457 and 340 days respectively. The results in other generations not included above seem to indicate no correlation between age and weight lost during lactation.

When the average results of all generations in each group are considered a better idea of the effect of the diets may be obtained. In group I the average age of the mothers of the 212 litters nursed was 302 days, the average per cent lost was 7.82, the average grams lost for each young nursed was 3.10 and the average size of the litter nursed was 4.6. In group II, 230 litters, age mother 300 days, per cent lost 8.03, grams lost per each young nursed 3.62 and size of litter nursed 4.70. Group III, 192 litters, age mother 321 days per cent lost 5.82, loss for one young 2.80 grams, and size of litter 4.46. Group IV, 64 litters, age mother 342 days, per cent lost 3.73, grams lost per one young nursed 1.69, average number nursed 4.69. In group V, 146 litters, age of mother 316 days, per cent lost 6.49, loss for each young 2.67 grams and average number in each litter nursed 5.31. The average age of the mothers in the different groups ranged between 300 and 342 days. The number of litters was sufficiently large to give fair average results. The average size of the litter nursed ranged between 4.46 and 5.31. The order of the efficacy of these diets

from greatest per cent loss to least was group II, I, V, III, and IV while the order for grams lost for each young nursed was II, I, III, V, and IV. These results show that the diets containing 18.2, 22.2 and 26.2 per cent protein enabled the mothers to nurse their young with less loss of weight than the 10.3 and 14.2 per cent groups. The average size of the litters nursed in group V was considerably larger than in any other group. Since the weight lost by the mother is closely related to the number of young nursed it is reasonable to assume that had these litters been no larger than in the other groups the per cent loss would have been less and may have altered the order of the group losses given above.

Offspring. No consistent results were noted as to effect of successive generations on the average number of young born by fertile females, on the average number of litters and on the average size of the litters. The generations in each group showed fluctuations in the average number born, average number of litters and average size of litter which we are unable to explain. The average size of the litters in each group showed the least changes. The largest and smallest average size litters in the generations of each group were: I, 5.15 and 4.17; II, 5.43 and 4.58; III, 4.88 and 4.07; IV, 5.50 and 3.74; and V, 6.97 and 4.29 respectively. The differences between the largest and smallest average litters in each group were: I, 0.98; II, 0.85; III, 0.81; IV, 1.76; V, 2.68. This shows that in the successive generations groups I, II and III were more nearly uniform in reproduction than groups IV and V.

The results of all generations in each diet group were combined to give the effect of these diets on the average age of fertile females at birth of young, the average number of young born, the average number of litters and the average size of each litter. In group I, 94 mothers at the average age of 324 days produced 2190 young, averaged 23.3 young each, average number of litters 4.78 and average size of litter 4.82. Group II had 102 mothers at the average age of 328 days produced 2142 young, averaging 20.8 young each, 4.43 litters with an average size of 4.73. In group III, 84 mothers at the average age of 377 gave birth to 1448 young, averaged 17.4 young each, had an average of 3.89 litters averaging 4.43 each. There were 46 mothers in group IV which at the average age of 327 days produced 636 young, averaged 13.8 young each, had an average of 2.93 litters of 4.71 each. In group V there were 74 mothers which at the average age of 346 days gave birth to 1381 young, averaged 18.6 young and 3.41 litters each with an average size of 5.48. The average ages varied only 53 days from the least of 324 days in group I to 377 days in group III. The order of the average number of young born from greatest to least was group I, II, V, III and IV with a range from 23.3 to 13.8. The order of the average number of litters from greatest to least was group I, II, III, V and IV. The average size of the litters from largest to smallest was group V, I, II, IV and III. This shows that the groups receiving

10.3 and 14.2 per cent protein in the diet surpassed all other groups in the average number of young and litters born and with the exception of group V, in the average size of the litters. That diets containing 18.2 or higher per cents of protein were detrimental to reproduction seems a justifiable conclusion.

When the average of the first, last and largest litters and the average age of the parents at birth of these litters in successive generations were considered we found the results quite irregular. There was, however, a noticeable tendency toward a reduction in size of the first and last litters and, with the exception of groups IV and V, in the largest litters in succeeding generations of each group. Further generations would be necessary to determine if this were a true indication of results. The average ages of the parents at birth of these litters in the succeeding generations of each group were so variable that no conclusions could be made.

To determine the effect of the different diets on the average size of the first, last and largest litters and the ages of the parents at birth of these litters we have combined the results of all generations in each group. This shows that in group I there were 86 pairs and the average ages at birth of first, last and largest litters of the males and females were 197 and 184, 503 and 443, 240 and 239 days respectively; group II, 90 pairs, 211 and 219, 488 and 448, 274 and 258 days; group III, 80 pairs, 236 and 219, 455 and 435, 282 and 266; group IV, 45 pairs, 255 and 275, 471 and 419, 306 and 326; group V, 63 pairs, 220 and 226, 421 and 423, 268 and 272 days respectively. The order of age at first litter from youngest to oldest was, males, I, II, V, III and IV; females, I, II, III, V and IV. At last litter, males, V, III, IV, II and I; females, IV, V, III, I and II. At largest litter, males I, V, II, III and IV; females I, II, III, V, and IV. This shows that both sexes in groups I and II commenced reproducing at an earlier age and continued to an older age than the other groups. The average sizes of the first litters produced by the males and by the females were group I, 5.65 and 5.57; II, 5.67 and 5.72; III, 4.84 and 5.07; IV, 4.67 and 4.72; V, 6.25 and 6.08 respectively. At last litter, I, 3.58 and 3.47; II, 3.79 and 3.93; III, 4.48 and 4.26; IV, 4.40 and 4.35; V, 4.82 and 4.70 respectively. The average size of the largest litter in each group was group I, 7.05; II, 6.84; III, 6.38; IV, 6.43; V, 7.40.

The order of the size of the first litters produced by the males from largest to smallest was group V, II, I, III, and IV; by the females, V, II, I, III, IV. A similar order for the last litters was, males, V, III, IV, II, I; females V, IV, III, II, I respectively. The order for the largest litters was V, I, II, IV, III. The average order in the series of litters when the largest litter appeared was slightly after the second in group I and between the first and second in all the other groups. The averages were I, 2.11; II, 1.75; III, 1.80; IV, 1.79; V, 1.69.

These figures show that for the production of large litters group V diet

was most efficient and was followed closely by groups II, I, III and IV in decreasing order.

Sex ratio of offspring. The sex ratio of the different generations in each group showed wide variations which in general indicated no effect of this number of generations on this phase of reproduction.

When the number of offspring of all the generations in each diet group is considered the effect of these diets on sex ratio can be determined. These ratios have been computed on the basis of the number of males to 100 females. Most investigators have found for the rat this to be approximately 106 males to 100 females. This ratio did not obtain in any of our groups. The following sex ratios were found at birth of young. In group I, 2230 young the ratio was 95.4:100; group II, 2173 young, 103.6:-100; III, 1467 young 95.3:100; IV, 657 young, 94.5:100; V, 1406 young, 93.3:100. Group II was the only one which approached the normal ratio. The ratio in the other groups decreased slightly in the order of I, III, IV and V.

The sex ratios of the offspring which were weaned was as follows: group I, 1203 young 97.1:100; II, 1494 young 105.1:100; III, 1067 young 101:-100; IV, 451 young 104:100; V, 889 young 91.6:100. The number of young which died before weaning and their sex ratio in each group was: I, 1027 young 93.5:100; II, 679 young, 100.4:100; III, 396 young, 80:100; IV, 242 young, 87.6:100; V, 417 young 97.5:100.

These results show that the pre-natal effect of these diets was more harmful to the males than the females and that the amount of harm in the different groups from greatest to least was in the order of V, IV, III, I and II. During the nursing period the order was V, I, III, IV and II. The group fed a diet containing 14.2 per cent protein approached most nearly the normal sex ratio both in young born and young which survived to the weaning age.

Mortality of offspring in each generation from birth until weaned. The average per cents of mortality of the young during the nursing period showed wide ranges in the generations of each group. In group I it was from 22.6 to 72.0; II, 27.5 to 55; III, 11.1 to 48.5; IV, 9.1 to 42.2; and V, 11.3 to 57.1. The greatest death rate was in the last generation in all the groups except group III which was greatest in the first generation. The percentage of deaths in the generations of each group, however, showed no definite indication of any kind as to an effect of this number of generations.

Where the results of all the generations in each group were considered we found that during the nursing period the average per cent of total mortality and the order of the groups from least to greatest death rate were: III, 1467 young, 27 per cent; V, 1406 young, 29.6 per cent; II, 2173 young, 31.1 per cent; IV, 657 young, 36.3 per cent; I, 2230 young, 46.1 per cent. The greatest mortality occurred during the first five or six days

following birth. From this time on there was a gradual decrease in the death rate during the nursing period. The per cent of mortality of the females was slightly greater than that of the males in all groups except group V in which they were 0.6 per cent less than the males. The average per cents of mortality during the nursing period for the males and for the females in each group were: I, 45.6 and 46.5; II, 29.3 and 31.6; III, 24.6 and 29.2; IV, 35.4 and 38.2; V, 30.4 and 29.0 per cent respectively. Since these averages represent relatively large numbers of young born, certain conclusions seem to be justifiable. The mortality of the nursing females is generally greater than that of the males. The diet containing 10.3 per cent protein (group I) was most lacking in growth and maintenance promoting ingredients than any of the other diet groups. The diet containing 18.2 per cent protein (group III) was most efficient of any of the groups in maintaining life and growth during the nursing period of the young.

Autopsies. At the death of the mated animals the age, sex, weight, length of body and tail, general external condition, tumors, etc. were recorded. Then a macroscopical examination of the internal organs was made and abnormal conditions noted. Finally the kidneys were removed and weighed separately. From these records we were able to determine the average life span, the weight at death, the relation of total kidney weight to body weight and in many cases the probable cause of death.

Kidneys. It is generally maintained that diets rich in protein cause hypertrophy of the kidneys. This is substantiated by our results. Since the kidneys of the male are heavier than those of the female we have grouped the results according to sex. In both sexes the average weight of the right kidney was heavier than the left. The combined weight of the kidneys in both sexes showed a tendency toward a reduction in weight in later generations but, since it was not a uniform or progressive reduction and showed fluctuations, the only statement that can be made is that the average weight of the kidneys in later generations was slightly less than in the earlier generations. Only a greater number of generations would prove if this tendency was a true indication of effect of succeeding generations.

To give the effect of the different diets on the kidneys we have averaged the average results of all the generations in each diet group. The number of males and the average weights in grams of the right and left kidney in each diet group were as follows: I, 39, 1.111 and 1.050; II, 40, 1.145 and 1.041; III, 44, 1.128 and 1.039; IV, 25, 1.293 and 1.257; V, 35, 1.311 and 1.226 grams respectively.

The results for the females were: I, 38, 0.948 and 0.858; II, 52, 0.995 and 0.894; III, 36, 0.986 and 0.923; IV, 23, 1.128 and 1.073; V, 46, 1.161 and 1.105 grams respectively. The combined weight of both kidneys in each group for the males and for the females was: I, 2.160 and 1.809; II, 2.186 and 1.908; III, 2.167 and 1.909; IV, 2.552 and 2.203; V, 2.534 and

2.266 grams respectively. These results indicate not only the difference in kidney weight in the two sexes but also the increased weight in the groups whose diets were richer in protein.

What explanation can be given for these differences? Since the size of the kidneys is correlated with the amount of nitrogenous waste material to be eliminated, which in turn is related to the size of the body, we naturally compare body weights of the two sexes. The average body weights at autopsy for the males and females in each group were: I, 178 and 158; II, 202 and 169; III, 189 and 170; IV, 218 and 164; V, 202 and 168 grams respectively. In order to show how much the heavier body weights of the males may explain their heavier kidney weight we have computed the per cent of body weight which the kidneys represent. These average per cents for the males and females in each group were: I, 1.213 and 1.143; II, 1.082 and 1.128; III, 1.146 and 1.119; IV, 1.170 and 1.341; V, 1.254 and 1.346 per cent respectively. With the exception of group I there was almost a gradual increase in kidney structure with the increase in the protein content of the diet. The exception in group I may possibly be explained by the considerably older age of both sexes of this group at death. With advancing age the rat becomes more and more decrepid and shrunken in appearance due to the utilization of adipose tissue and to general atrophy. This greatly reduces the body weight. The atrophy of the vital organs is at a minimum and their weight would represent a greater percentage of the total reduced body weight than in younger animals. The age at death would therefore modify the ratio of kidney weight to body weight. The average ages of the males and females at autopsy in the groups were: I, 940 and 965; II, 824 and 883; III, 762 and 841; IV, 788 and 920; V, 799 and 819 respectively. These differences in age, in our opinion, play an important part in modifying the ratios given above. To get a true effect of these diets on the relative weight of the kidneys animals of the same age should be used.

Life span and weight at death. The results of the different generations in each group are such that no conclusions can be made. The average life span and weight varied only within normal limits in both sexes and indicated no tendency in any manner. Only the average results of all generations in each group will be considered. Since sex influences body weight the results of each sex will be given separately. The animals here involved consisted only of those which had been mated. The other offspring had to be discarded prior to normal death.

The numbers of males and females mated in each group were: I, 121 and 113; II, 121 and 121; III, 128 and 117; IV, 97 and 114; V, 105 and 109 respectively. The average weights at death for males and females were: I, 191 and 140; II, 212 and 186; III, 207 and 161; IV, 201 and 162; V, 214 and 172 respectively. The average ages for males and females were: I, 775

and 852; II, 787 and 789; III, 755 and 797; IV, 692 and 687; V, 696 and 724 respectively. With the exception of group IV, the females had a longer life span than the males, but the males were much heavier than the females in all groups. This is a normal sex difference. Some of the animals died prematurely often at an early age. The ranges in the age at death for the males of each group were: I, 309 to 1334; II, 269 to 1559; III, 168 to 1172; IV, 228 to 1142; V, 159 to 1327. For the females they were: I, 110 to 1334; II, 147 to 1258; III, 209 to 1379; IV, 212 to 1503; V, 145 to 1214.

The order of life span from longest to shortest was, males: II, I, III, V, IV; females: I, III, II, V, IV. This shows that the diets containing the highest per cents of protein were not conducive to a long life. The order of the weights at death from greatest to least was: males, V, II, III, IV, I; females, II, V, IV, III, I. Since the weight at death is influenced by age these orders of weight are not so indicative. Nevertheless it is interesting to note that both sexes of group I were the lightest and of groups V and II the heaviest.

Cause of death. Only the animals of the mated pairs which were autopsied were considered in this group. Abnormal conditions of the lungs and alimentary tract and the presence of ulcers and tumors were noted and the per cent of animals in which these abnormalities occurred was computed. Lesions of the lungs were most frequently found. These varied over wide limits from a slight congestion to a tubercular-like appearance. It is very probable that the dry and powdery food which all groups were fed had much to do with the lung infection. The alimentary tract was the next most commonly affected region. This consisted of congestion and dilatation due apparently to gas-forming bacilli. This dilatation was most frequently found in the large intestine and often caused an increase in diameter to several times the normal, making the wall almost transparent. Ulcers, sores and tumors were much less common. The females had a greater per cent of tumors than the males. There was no evidence of any consistant effect of successive generations. Only the combined results in each diet group will therefore be given.

The males showed the following per cents of abnormalities in the diet groups. I, 109 animals, average age 783 days, weight 189 grams, lungs 78.7, alimentary tract 47.7, ulcers 2.75 and tumors 2.75 per cent. II, 114 animals, average age 772 days, weight 209 grams, lungs 79.8, alimentary tract 57.8, ulcers 2.65 and tumors 6.14 per cent. III, 116 animals, average age 737 days, weight 185 grams, lungs 71.5, alimentary tract 51.7, ulcers 3.45, tumors 1.72 per cent. IV, 78 animals, average age 708 days, weight 198 grams, lungs 76.9, alimentary tract 60.2, ulcers 1.28 and tumors 2.56 per cent. V, 81 animals, average age 710 days, weight 203 grams, lungs 71.6, alimentary tract 53.0, ulcers 1.00 per cent and no tumors. The

order of disturbance in the males from greatest to least was as follows: lungs II, I, IV, V, III; alimentary tract, IV, II, V, III, I; ulcers, III, I, II, IV, V; tumors, II, I, IV, III, V. Although these results are somewhat irregular they seem to indicate that in the male the lower per cent protein diets are more favorable to lung trouble, ulcers and tumor growth and less favorable to digestive disturbances than diets richer in protein.

The females gave the following results: I, 103 animals, average age 813 days, weight 159 grams, lungs 70.8, alimentary tract 41.7, ulcers 2.91, tumors 14.55 per cent, II, 124 animals, average age 816 days, weight 182 grams, lungs 66.0, alimentary tract 47.5, ulcers 4.83, tumors 21.79 per cent. III, 104 animals, average age 777 days, weight 181 grams, lungs 67.3, alimentary tract 38.4, ulcers 2.88, tumors 23.08 per cent. IV, 93 animals, average age 738 days, weight 165 grams, lungs 73.1, alimentary tract 47.3, ulcers 4.29, tumors 8.60 per cent. V, 88 animals, average age 777 days, weight 170 grams, lungs 71.6, alimentary tract 51.2, ulcers 5.68, tumors 19.33 per cent. The tumors were mostly mammary, ovarian or uterine though some were miscellaneously placed. The order of disturbance in the females from greatest to least was as follows: lungs, IV, V, I, III, II: alimentary tract V, II, IV, I, III: ulcers V, II, IV, I, III; tumors III, II, V, I, IV.

Comparing the order of infection in these organs we note a lack of conformity of the sexes. We feel that the number of cases involved was too small to give accurate results. These observations, however, do indicate certain effects of these diets. In the lungs there is suggested a sex difference. In the males the groups having the higher per cents of protein had the least lung disturbance and ulcers while almost the reverse obtained in the females. With but slight differences the lesions of the alimentary tract were the same in each sex. Both sexes in general showed that the lower per cent protein groups had the greater number of tumors. These suggested indications of effect of the different diets might be more pronounced if a larger number of observations could have been made.

SUMMARY

1. The eruption of the incisor teeth was at an earlier age in group I and was delayed almost progressively with the addition of protein in the diet. There was a tendency toward delayed eruption in the later generations in each group.

2. The eyes opened at the earliest age in group V and at the oldest age in group I. Later generations tended to delay the opening of the eyes.

3. The order of the groups as to age at disappearance of the vaginal membrane from earliest age to latest was: III, 51.1 days; II, 55.2; V, 56.8; IV, 57.8 and I, 68.7 days. No consistent effect of successive generations was noted.

4. The average maximum weights of mated animals from greatest to

least was: males II, 314; V, 310; III, 297; I, 286; and IV, 274 grams. Females II, 264; III, 245; V, 244; IV, 237; and I, 231 grams. Group I required the greatest number of days to reach the maximum weight and group V the least in both males and females. The effect of successive generations on maximum weight was too varied to warrant any conclusion.

5. Group V attained the greatest stature (body length) and group I the least in both sexes. No definite effect of successive generations was noted.

6. The weight of the offspring at birth was greatest in both sexes of group III and was least in group I. There was a marked tendency toward less weight at birth in later generations.

7. The rate of growth of the offspring was least in group I and in general showed an increased rate with the addition of protein to the diet. A marked tendency toward increased rate of growth was noted in succeeding generations.

8. The per cent of sterility in both sexes was least in group II and greatest in group IV. No effect of succeeding generations was found.

9. The age at the birth of the first litter was youngest in group I. The order of the groups from earliest to latest age was for the males: I, II, V, III and IV. For the females it was I, II, III, IV, V. Results in different generations were too varied to warrant a conclusion.

10. The oldest age at last litter for both sexes was in group II. The order of groups for the males was: II, I, III, V and IV. For the females it was II, III, IV, V and I. No effect of successive generations was noted.

11. The reproductive span for the males was greatest in group I and was followed in order by II, V, III and IV. In the females it was longest in group II and was followed in a descending order by groups I, V, III and IV. Successive generations showed no definite effect.

12. The per cent loss in weight by mothers during lactation for each young nursed was least in group IV and greatest in group II. The order was IV, V, III, I and II. There was a marked decrease in weight lost in successive generations.

13. The average largest litter was formed in group V and the smallest in group III. The order was V, I, II, IV and III. Successive generations showed no consistent effect. The serial number of the largest litter ranged from 2.11 in group I to 1.69 in group V.

14. The average sex ratio of the offspring was below normal in all the diet groups. Group II approached most closely to the normal. The order was II, I, III, IV and V. The successive generations gave no consistent results.

15. The total mortality of the offspring from birth to weaning age was greatest in group I and least in group III. In most groups the mortality of the females was greater than that of the males. There was a tendency toward an increase in the death rate in later generations.

16. The average total weight of the kidneys at autopsy increased from

group I to V. They were heavier in the male than in the female. The average weight of the right kidney was heavier than the left in each sex. There was a marked reduction in weight in later generations. The ratio of kidney weight to body weight was greatest in both sexes of group V and least in group II males and group III females.

17. The average weight at death was greatest in the males of group V and the females of group II. No effect on death weight of successive generations was noted.

18. The average life span was longest in the males of group II and the females of group I. With the exception of group IV the females lived longer than the males. The results of the different generations were too variable to show any definite effect.

19. Lesions of the lungs and alimentary tract and tumors were the most common causes of death. Tumors were more prevalent in the females than in the males. The highest per cent of tumors in the males (6.14 per cent) was found in group II. The highest per cent in the females (23.08 per cent) was found in group III.

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